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THE ETIOLOGY OF VISCERAL LARVA MIGRANS

I. DIAGNOSTIC MORPHOLOGY OF INFECTIVE SECOND-STAGE *TOXOCARA* LARVAE*

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INTRODUCTION

A syndrome characterized by eosinophilic leukocytosis in association with transitory pulmonary infiltration has been recognized for many years. The etiology in the majority of instances has remained a matter of conjecture. Perlingiero and György (1947) presented a case in which a young child exhibited focal necrosis of the liver in addition to eosinophilia and pulmonary changes. Zuelzer and Apt (1949), Mercer *et al.* (1950) and Behrer (1951) reported similar cases in young children. Nematode larvae, identified as *Ascaris lumbricoides,* were discovered in liver lesions in the cases of Mercer and Behrer. However, the identifications were made only through association of the larvae with concomitant intestinal ascariasis and not by means of known larval morphology. Following the identification by Beaver *et al.* (1952) of a canine or feline nematode larva (*Toxocara* sp.) in a liver biopsy from 1 of 3 children exhibiting similar symptons, considerable attention has been directed to host-foreign nematodes as agents capable of producing disease in man. Beaver *et al.* designated this eosinophilia-hepatomegaly syndrome, caused by visceral invasion of immature nematodes adapted to lower animals, as visceral larva migrans.

In 1953 Milburn and Ernst presented an analysis of 15 reported cases of this syndrome observed in young children. Larval nematode etiology was reported in 4: *Ascaris lumbricoides* in 2 cases and *Toxocara* in 2 others. The case histories and clinical evidence in the remaining 11 children were preponderantly in favor of larval nematode infection. Wilder (1950) reported the finding of nematode larvae in 24 of 46 eyes which had been enucleated, in most instances following a clinical diagnosis of retinoblastoma. In 9 of the eyes the worms were identified as third-stage hookworm larvae. Beautyman and Woolf (1951) reported a larva tentatively diagnosed as *Ascaris lumbricoides* in a human brain specimen. The patient's death resulted from poliomyelitis; no symptoms were ascribed to nematode infection. Dent and Carrera (1953) presented 4 clinical cases in which *Toxocara*

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canis was identified in 1 liver biopsy and in 1 complete autopsy. The autopsy, reported in greater detail in another paper (Dent *et al.*, 1956), described the heaviest infection yet studied; a minimum of 50,000 larvae were present in the body. In an earlier autopsy reported by Brill *et al.* (1953), a *Toxocara* larva was identified in sections of the lung.

The preliminary study by Beaver *et al.* (1952) indicated that both *T. canis* and *T. cati* produce in white mice infections which resemble in many respects those of their 3 cases of visceral larva migrans. Second-stage larvae recovered from mice as long as 3.5 months after infection were indistinguishable from the infective larva in the egg. Furthermore, the larva observed in a human biopsy had size relationships similar to those of the infective second stage. As regards the migratory pathway of initial infection, different investigators have established that *Toxocara* larvae utilize a liver-lung route in both the normal and the abnormal host (Fülleborn, 1921; Hoeppli *et al.*, 1949; Sprent, 1952). Embryonated eggs introduced into the stomach hatch within several hours in the stomach or small intestine. The larvae migrate to the liver in large part by way of the portal system, where they may either rupture the venous sinusoids and enter the hepatic parenchyma or migrate directly from the hepatic veins into the inferior vena cava. Other larvae entering the intestinal lymphatics probably pass into the portal circulation by way of the venous capillaries of the mesenteric lymph nodes. Reaching the lungs via the pulmonary arteries, the larvae either rupture the alveolar capillaries, ascend the bronchial tree and trachea to enter the pharynx where they are swallowed or they may enter the systemic circulation by a mechanism not completely understood. Experimental evidence suggests that the latter pathway is almost invariably utilized in the experimental abnormal host.

The present study was undertaken to provide a detailed morphologic description of *T. canis* and *T. cati* larvae in the mouse to facilitate reliable diagnosis of the larvae in biopsy and necropsy specimens.

MATERIALS AND METHODS

A combined sedimentation-zinc sulfate flotation technic was employed to concentrate *Toxocara* eggs from the feces of naturally infected dogs and cats. Incubation of the eggs was carried out at room temperature in a 0.5% solution of formalin. The period required for complete embryonation varied from 2 to 7 weeks, depending upon the depth and density of the suspension and the temperature. Before inoculation the embryonated eggs were washed with tap water to remove the formalin. After light ether anesthesia mice were inoculated by direct introduction of infective eggs into the stomach, using a 2 cc syringe with an attached small-diameter catheter. The number of eggs given each mouse was arbitrarily adjusted to the predetermined interval between infection and sacrifice. Mice held for longer periods of time were given smaller infective doses; the inoculum varied from 200 to 12,000 eggs. Mice were sacrificed 24 hours to 6 months following infection. Tissues to be sectioned were fixed in Bouin's solution and standard procedures were followed in dehydration, clearing and embedding in paraffin. Serial sections 5 microns thick were stained with hematoxylin and eosin. Selected sections were stained by the iron alum-picric acid-hematoxylin method of Goldman (1951) or by the one-step trichome method of Gomori (1950).

Larvae were studied in the living state while immobilized in fresh egg albumin or polyvinyl alcohol, or by pressure of a coverslip. All measurements were made of heat-killed larvae. Tissue digestion permitted recovery of both free and encapsulated larvae which were vigorously motile and morphologically unaltered. Macerated tissue, not in excess of 2 gm, was suspended in 20 cc of freshly prepared 1.5% pepsin solution adjusted to pH 2 with hydrochloric acid, and incubated over night at 37° C. Unstained larvae were studied in wet mounts using standard achromatic and apochromatic systems. The use of phase contrast microscopy as well as the examination of stained specimens proved unsatisfactory.

RESULTS

General Considerations

The average length of 150 fully embryonated *T. canis* larvae pressed from the egg was 404 microns with a range of 360 to 434 microns. A range in length of 357 to 445 microns was recorded for 229 larvae from various tissues of mice (liver, skin, brain, lung, kidney, and heart) infected for 24 hours to 180 days. The average width of various groups, 18 to 20 microns, displayed little variation. Significant growth or internal differentiation of the larvae was not observed in any tissue of the mouse as long as 180 days after inoculation, the greatest interval recorded in this study. In like manner, *T. cati* larvae in the mouse remained developmentally identical with the infective second-stage. Larvae pressed from eggs and those recovered from tissues of infected mice had similar ranges in length which corresponded very closely with those of *T. canis*. Infective-stage *T. canis* and *T. cati* have almost identical morphology, differing only in their maximum diameter which in *T. canis* varied from 18 to 21 microns while in *T. cati* the range was from 15 to 17 microns (Table I).

TABLE 1.—*Mean dimensions, in microns, of* Toxocara canis *and* T. cati *larvae pressed from the egg and recovered from mouse tissues*

Species	Source	No. of larvae	L*	W	Esophagus	Ex. pore	Anus
T. canis	Egg	150	404	18	153	74	38
	Liver	85	414	19	150	79	38
	Lung	33	396	18	154	77	36
	Brain	74	416	18	155	78	38
	Kidney	18	405	19	151	78	38
	Heart	10	390	19	156	77	37
	Skin	9	410	18	150	75	40
T. cati	Egg	131	404	16	155	75	39
	Liver	79	411	16	153	77	39
	Lung	60	410	16	155	77	39
	Brain	43	404	16	157	77	39
	Kidney	4	394	16	—	74	38
	Heart	5	413	16	153	77	39
	Skin	10	414	16	153	76	40

* Length; W, width; Esophagus, length of esophagus; Ex. pore, mouth to excretory pore; Anus, anus to tip of tail.

Morphology of Living and Heat-Killed Larvae

When pressed from eggs, infective second-stage larvae are highly active and essentially identical with larvae recovered from experimentally infected mice and with those from tissues of one human autopsy (Dent *et al.*, 1956). They are cylin-

drical with the body having almost parallel lateral margins in the optical plane (Plate I, Figs. 1, 2). The anterior quarter of the body tapers evenly to a 3-lipped, subterminal, dorsally inclined mouth. Slightly anterior to the lips a sharp, spine-like cuticular thickening forms the ventral margin of a shallow buccal capsule. The posterior end of the larva is more abruptly tapered, commencing anterior to the termination of the intestine and continuing to form a rather slender attenuated tail. Under low magnification the larvae can be divided roughly into 2 portions, a clear structureless esophageal region occupying less than one-third of the total length and a more opaque intestinal region filled with refractile globules terminating about 60 microns anterior to the tip of the tail. The excretory pore is found in the mid-ventral line at about the middle of the esophagus. The anal pore, likewise in the mid-ventral line, is found about equidistant from the tip of the tail and the termination of the intestine. Two cuticular thickenings, the lateral alae, are seen as refractile lines extending along the sides of the larva, terminating about 20 microns from either extremity.

Buccal Apparatus. The cup-shaped buccal capsule has a complete external margin with cuticle continuous with that of the body wall and with the cuticular lining of the esophagus (Plate I, Fig. 11). The prominent ventral margin of the capsule results from a sharp infolding of thickened cuticle. The cuticle of the dorsal lip is applied closely to its external surface. That of the 2 sub-ventral lips is separated from the external margin and projects slightly forward.

Esophagus. The esophagus extends for approximately one-third of the total length of the larva (Plate I, Fig. 1). A central cuticular lining is seen as a refractile line from the buccal capsule to the intestine. The esophagus is grossly divisible into four regions: a slender procorpus 20 microns in length; a thickened metacorpus 35 microns in length and 5 microns in width; a slender elongate isthmus equivalent in length to the procorpus and metacorpus together, and a terminal bulb 20–25 microns in length and 6–10 microns wide. Little cellular investment was observed for the procorpus; it consists of little more than a naked cuticular tube. The metacorpus originates with a single layer of cells surrounding the cuticular lining of the esophagus and extends to the nerve ring, 5 to 7 microns anterior to the excretory pore. The isthmus, having a decreased diameter, continues through the center of the nerve ring and terminates in a small globular enlargement, the bulb.

Nerve Ring. The nerve ring appears as a clear or homogeneous structure with an outline made visible by the regular limitation along its margins of the cellular elements of the mid-esophageal region (Plate I, Fig. 1). Intimately associated with the nerve ring and surrounding most of the esophagus are large numbers of ganglionic nuclei which form a sheath separating part of the procorpus and all of the metacorpus and isthmus from the body wall.

Esophageal Glands. The dorsal esophageal gland forms a well-defined structure, whereas the 2 subventral glands are not obvious. The dorsal gland consists of an elongated cell extending into the procorpus and having its nucleus embedded in the most posterior portion of the dorsal sector of the esophageal bulb (Plate I, Fig. 1). The nucleus, 4 to 6 microns in diameter, is clearly different from the muscle nuclei of the bulb. The cytoplasm of this cell contains characteristic refractile granules which permit its differentiation from associated structures. The subventral glands lack these granules and their nuclei appear similar in size to muscle nuclei of the bulb.

Excretory Cell. The unicellular excretory apparatus of *Toxocara* is typical of the ascarid group, a shortened H-type as described by Chitwood (1950). The cell body is greatly elongated, having 2 lateral columns extending posteriorly on either side of the intestine and anteriorly some distance beyond the esophageal bulb (Plate I, Fig. 1, 2). At this position the esophageal isthmus is forced dorsad and the surrounding nuclear sheath is disorganized. As the esophageal bulb is approached, the isthmus continues to be forced dorsad, reaching its maximum displacement at the position of the nucleus of the excretory cell, located just anterior to the origin of the bulb. In second-stage larvae pressed from the egg, the lateral columns of the excretory cell terminate 90 to 100 microns posterior to the esophageal bulb and their central excretory canaliculi are not well differentiated. However, in larvae recovered from mice as early as 24 hours following infection the canaliculi are well defined, and the excretory columns extend 130 to 150 microns posterior to the esophageal bulb. The posterior columns join 5 to 10 microns anterior to the termination of the esophagus and on its ventral surface. The united cell bifurcates anterior to its nucleus forming columns 10 to 15 microns long. The canaliculi unite just posterior to the nucleus but separate immediately, sending an extension forward into each short projection of the cell body. The convoluted terminal excretory tubule joins the canaliculi at their point of junction.

Intestine. The intestine is without apparent lumen and consists of 7 elongate cells arranged in linear fashion (Plate I, Figs. 1, 2). The cytoplasm of the cells contains opaque neutral fat globules, the number being reduced in old larvae either from the egg or from infected animals. The intestine is strongly compressed laterally and forced dorsad by the excretory columns. Posterior to the columns it expands and fills the body cavity. The seventh intestinal cell terminates 20 to 25 microns anterior to the anal pore to which it is connected by a thin cuticular tube.

Genital Primordium. The genital primordium is a small lens-shaped mass between the intestine and the ventral body wall slightly anterior to the mid-intestinal level. It consists of 4 cells which are not individually discernible (Plate I, Fig. 1).

Interpretation of Larvae in Tissue Sections

During dehydration and clearing, there is usually more shrinkage in length than in diameter. *T. canis* larvae may exhibit a range in maximum diameter of 14 to 20 microns. Generally, however, the diameter remains 18 to 20 microns. Reconstructed larvae range in length from about 290 to 350 microns. The relationship of *T. cati* and *T. canis* larvae remains the same; they have similar ranges in length but *T. cati* is more slender, being 12 to 16 microns in diameter. Dimensions of internal structures, with the exception of the dorsal esophageal gland nucleus and the nucleus of the excretory cell, are correspondingly less.

Esophageal Region. In longitudinal sections the structure of the esophagus is quite often obscured by numerous basophilic ganglionic nuclei. Only median sagittal or frontal sections less than 7 microns thick will demonstrate diagnostic characters in this region. The characteristic buccal structure is not often seen in sections but the prow-like shape of the anterior tip may be evident (Plate IV, Fig. 1). The acidophilic nerve ring is usually seen in longitudinal sections since ganglionic nuclei are absent at this level (Plate IV, Fig. 3). It is found at approximately the mid-portion of the esophagus. The nucleus of the excretory cell

and that of the dorsal esophageal gland often appear to occupy nearly one-third the diameter of the larva. It is evident that they do not undergo reduction in size during dehydration and clearing comparable with that of associated structures. The nucleus of the excretory cell is regularly round and vesicular with a diameter of 5 to 7 microns (Plate I, Fig. 5; Plate III, Fig. 19). The thick nuclear membrane is lined internally with irregularly arranged chromatin granules. A round nucleolus, 2-3 microns in diameter, is situated eccentrically in contact with the nuclear membrane. The nucleus of the dorsal esophageal gland is more difficult to demonstrate since it is embedded in the nuclear mass of the esophageal bulb. However, it seems to have internal organization similar to that of the nucleus of the excretory cell (Plate I, Fig. 7; Plate III, Fig. 21). It has a diameter of 4-5 microns with a correspondingly smaller nucleolus. Transverse sections at the level of the procorpus show loosely organized ganglionic nuclei surrounding the esophagus which is of small diameter and devoid of nuclei (Plate II, Figs. 2-5). Transverse sections through the metacorpus and isthmus show 3 concentric layers of nuclei (Plate I, Figs. 3, 4; Plate II, Figs. 6-12; Plate III, Figs. 13-18). Hypodermal and muscle nuclei are in close association with the body wall and constitute the most peripheral layer. The middle layer of ganglionic nuclei forms a well-defined circular sheath surrounding the esophagus and its nuclear elements. Lateral alae are prominent at these and at more posterior levels. Transverse sections at the level of the bulb are characterized by a central basophilic mass, the bulb, surrounded by ganglionic, muscle and hypodermal nuclei (Plate I, Figs. 6, 7; Plate III, Figs. 19-21; Plate IV, Figs. 4-6). Near the junction of the isthmus and bulb the excretory cell forces the esophagus dorsad (Plate I, Fig. 5; Plate III, Figs. 17-19; Plate IV, Fig. 4). The vacuolated cytoplasm of this cell may either fill the body cavity in an irregular manner (Plate III, Figs. 17-18) or, posterior to the nucleus, 2 lateral excretory columns may be resolved (Plate I, Figs. 6-7; Plate III, Figs. 20, 21; Plate IV, Fig. 5). Since the single excretory cell with the two lateral columns forms a very flexible organ, its semi-fluid contents shift freely from one portion to another. The structure of the organ at any level, therefore, depends upon the position of the larva at the interval of fixation.

Intestinal Region. Longitudinal sections of the intestinal region present similar features throughout its length. The gut cells and the posterior excretory columns frequently appear as an unorganized, vacuolated, eosinophilic mass (Plate IV, Fig. 2). Discrete membranes are usually not evident between the separate intestinal cells. The seven regularly spaced intestinal nuclei are the only basophilic masses of nuclear size within the central body cavity (Plate I, Fig. 9; Plate IV, Fig. 2). The cytoplasm of the gut cells often contains minute basophilic granules which aid in distinguishing between intestine and excretory columns. In sagittal or frontal sections one or both excretory columns may appear to occupy the entire body cavity since the intestine is strongly compressed and stains poorly. In transverse sections the intestinal region may be divided into 3 zones, the first 2 occupying about one-third of the total body length. At the first level the excretory columns have their greatest diameter and fill nearly all of the body cavity (Plate I, Fig. 8). The gut cells are compressed dorsad into a small triangular area. At the second level the columns are reduced in diameter and the intestine extends into the ventral body cavity (Plate IV, Fig. 7). The third level commences with the termination of the excretory columns. Here the intestine fills

the body cavity and consists of the terminal 3 cells (Plate I, Fig. 9; Plate IV, Fig. 9). Lateral alae are conspicuous at all intestinal levels. The excretory canaliculi, found in larvae as early as 24 hours following infection, appear as central condensed portions of the posterior columns (Plate I, Figs. 6-8; Plate III, Figs. 21-24). They extend the full length of each column (Plate I, Fig. 2). At levels posterior to the intestine the body cavity is filled with nuclei which defy interpretation (Plate I, Fig. 10; Plate IV, Fig. 10).

Body Wall. .The body wall is formed by an outer noncellular cuticle, a median hypodermal layer and an inner layer of somatic muscle cells. The cuticle is evident only in transverse sections of the lateral alae; in other areas it is closely adherent to the hypodermis where the 2 layers cannot be separately distinguished since they stain similarly with eosin (Plates III, IV). Dorsal, ventral and lateral chords are not evident, although ventral-line nuclei are quite prominent (Plate III, Figs. 20-24). The medullary portion of each somatic muscle cell consists largely of a dense nucleus which projects into the body cavity (Plate III, Fig. 20).

DISCUSSION

No evidence has been found that in experimentally infected mice *Toxocara* larvae undergo growth or differentiation, with the exception of a slight development of the excretory cell. Hoeppli *et al.* (1949) reported that larvae of *T. canis,* like *Ascaris lumbricoides* and *Parascaris equorum,* undergo an increase in both length and width in the liver of mice within 2 weeks following infection. The extent of this growth was not mentioned.

In the present study measurements indicate that wide variation in length may occur in larvae of either *T. canis* or *T. cati* recovered by digestion of tissues from mice. However, a similar variation in length was found in second-stage larvae pressed from eggs. Differences in the length of the larvae were not found to be associated with the duration of infection or with the tissues from which the larvae were recovered. It may therefore be concluded that the differences in size of larvae recovered from mice resulted not from growth in the tissues but from the inherent variation in size of the infective larvae.

T. canis and *T. cati* larvae could be distinguished from each other only by their difference in equatorial width. This was the most constant of the measurements made. *T. canis* larvae regularly had widths of 18 microns or greater, while *T. cati* were never found to exceed 17 microns, with a weighted average of 16 microns.

The report of Wilder (1950), describing immature hookworms in human occur in larvae of either *T. canis* or *T. cati* recovered by digestion of tissues from *Toxocara* in mouse tissue. Through the courtesy of Brig. General Elbert De Coursey, then Director, Armed Forces Institute of Pathology, Walter Reed Army Medical Center, Washington, D. C., sections of 5 larvae in enucleated eyes from the Eye Registry of the Institute were made available for study. All of the larvae apparently were alive in the tissue at time of fixation. Sufficient material permitted the identification of second-stage *T. canis* in 4 eyes (specimen nos. 293903, 69623, 298563, 689806). In the fifth specimen, no. 189761, only 1 sagittal section of larva fragment was found. However, on the basis of identifiable characters, it was possible to exclude *Ascaris lumbricoides,* hookworm and *Strongyloides* larvae from the diagnosis. Most probably this larva is also *Toxocara.*

Mercer *et al.* (1950) described 4 nematode larvae, identified as *Ascaris lumbricoides,* in a liver biopsy from a child. A reconstruction of one larva measured 110 by 20 microns; probably less than one-half was found. A photomicrograph of a sectioned larva revealed little structure other than lateral longitudinal alae. However, as indicated by Beaver *et al.* (1952), these larvae were probably *Toxocara* sp. since infective eggs of the same species, confused with those of *A. lumbricoides* by the authors, were recovered from a dirt basement where the child played.

In the case presented by Behrer (1951), one larva identified as *A. lumbricoides,* was found in serial sections of a liver biopsy. A photomicrograph of the coiled larva, sectioned transversely in 3 regions posterior to the esophagus, was included, although no measurements were given. The evident structures were similar to those of *Toxocara.* Two lateral extensions of the excretory cell with very prominent central excretory canaliculi were present in one section. The intestine, as seen at a lower level, had no lumen and appeared to have a single centrally placed nucleus. Lateral alae were not evident, but the body wall of 2 sections of intestinal levels was incomplete. The body diameter could only be approximated, but was 20 microns or slightly less.

Beautyman and Woolf (1951) described an ascarid larva recovered in serial sections of brain from a child at autopsy. The reconstructed larva measured 310 by 15-20 microns. However, it was stated that because the entire tail was not recovered, the total length would probably approach 350-360 microns. The esophagus was indicated in a reconstruction as a muscular rhabditoid form, 95 microns long, 7 microns wide, and with a bulb 10 microns wide. Lateral longitudinal alae were presented in the reconstruction but could not be determined in the photomicrograph. All of these characters suggest *Toxocara.*

Morphologic descriptions of other tissue-invading larvae, presented in another paper (Nichols, 1956), make possible the specific differentiation between *Toxocara* and certain other nematodes of similar size on the basis of transverse sections of the mid-gut level. For example, the nematode observed in sections of a liver biopsy by Milburn and Ernst (1953) can be stated with certainty to be a second-stage *T. canis* larva on the basis of a photomicrograph of a single transverse section. The diagnostic features are as follows: diameter between 17 and 21 microns; prominent, single lateral alae; single intestinal cell; large, well defined posterior excretory columns occupying a greater proportion of the body cavity than the intestine.

Knowledge of comparative larval morphology simplifies the diagnosis of *Toxocara* and makes unnecessary elaborate reconstructions. Reference to corresponding structures of other nematode larvae clearly illustrates the morphologic specificity of each form. In most cases, attention to the maximum width, lateral alae, excretory cell and intestine is adequate for specific diagnosis of larval nematodes studied thus far in tissue.

SUMMARY

Toxocara canis and *T. cati* larvae recovered from experimentally infected mice were studied *in toto* and in serial sections of infected organs. Measurements of 229 *T. canis* and 206 *T. cati* larvae from various tissues from 24 hours to 180 days following infection indicate that no development of either species occurs in the mouse, with the exception of slight growth of the excretory cell. *T. canis* larvae in the mouse are morphologically identical with those recovered in human infection.

The length of both species of *Toxocara* pressed from the egg and recovered from experimental infections in mice, and of *T. canis* from one human autopsy, varied around 400 microns. The width of *T. canis* varied from 18 to 21 microns. For both larvae average measurements were: length of esophagus, 150 microns; distance from oral spine to excretory pore, 75 microns; and distance from anus to tip of tail, 38 microns.

Larvae in tissue specimens regularly exhibit reduction in length amounting to approximately one-fifth of their heat-killed dimensions following standard procedures of dehydration and clearing in preparation for sectioning. Reconstructed larvae of both species have average lengths of 320 microns. *T. canis* has a range in maximum diameter of 14 to 20 microns and *T. cati* 12 to 16 microns.

The diagnosis of *Toxocara* larvae may be made on the basis of one good transverse section of the mid-gut level. Longitudinal sections or transverse sections at levels other than the intestine provide fewer species-exclusive characters and only infrequently permit identification from a single section.

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PLATE I

EXPLANATION OF PLATES

(All drawings were made with the aid of a camera lucida)

FIG. 1. Second-stage *Toxocara canis* larva pressed from the egg showing structures evident in lateral view. Heat-killed, unstained. ×780.

FIG. 2. Second-stage *T. canis* larva 4 weeks in mouse brain. Ventral aspect. Heat-killed, unstained. ×300.

FIGS. 3–10. Transverse sections of a *T. canis* larva 4 weeks in mouse brain at levels indicated in Fig. 2. H. and E. ×1080.

FIG. 11. Buccal apparatus and procorpus of a second-stage *T. canis* larva pressed from the egg. Heat-killed, unstained. ×1600.

A, anus; DES, dorsal esophageal gland; DESN, nucleus of dorsal esophageal gland; EB, esophageal bulb; EC, excretory column; ECAN, excretory canaliculus; EN, nucleus of excretory cell; EP, excretory pore; ET, excretory tubule; GN, ganglionic nucleus; GP, genital primordium; INT, intestine; INTN, nucleus of intestinal cell; IS, isthmus; MC, metacorpus; NR, nerve ring; PC, procorpus.

PLATE II

A *Toxocara canis* larva 4 weeks in mouse brain. Serial 5 micra sections of the esophagus. H. and E. Photomicrographs, ×1800.

FIG. 1. Oral level showing 1 dorsal and 2 subventral lips.

FIGS. 2–5. Levels of the procorpus. Ganglionic and muscle nuclei surround the central esophagus.

FIGS. 6–9. Levels of the metacorpus. Ganglionic nuclei form a continuous sheath surrounding the esophagus.

FIGS. 10–12. Levels of the isthmus. The nerve ring is shown in Fig. 11.

PLATE III

Continuation of serial sections of the larva shown on Plate II. H. and E. ×1800.

FIGS. 13–18. Levels of the isthmus. Ganglionic nuclei surround the esophagus. The body of the excretory cell is immediately ventral to the esophagus in Figs. 16–18.

FIGS. 19–21. Levels of the bulb. The nucleus of the excretory cell can be seen in Fig. 19. The excretory cell has divided into 2 columns in Figs. 20 and 21.

FIG. 22. Level of the intestine immediately posterior to the esophageal bulb. The intestine is centrally compressed and the 2 excretory columns occupy prominent positions.

FIGS. 23, 24. Levels of the intestine near the mid-region where it is compressed dorsad and the excretory columns fill the body cavity. Central canaliculi are prominent.

PLATE IV

FIG. 1. Median sagittal section of the anterior end of a *T. canis* larva 4 weeks in mouse brain. The characteristic oral spine, buccal apparatus and esophageal isthmus are well defined. H. and E. ×1800.

FIG. 2. Median sagittal section of the intestinal region of a *T. canis* larva 4 weeks in mouse brain. A single intestinal cell nucleus is centrally positioned. H. and E. ×1800.

FIG. 3. Median sagittal section of the esophageal region of a *T. canis* larva 5 months in mouse brain. The procorpus, metacorpus and isthmus extend as a central column surrounded by ganglionic nuclei except at the level of the nerve ring. H. and E. ×1800.

Transverse sections of *T. cati* larvae in mouse lung (Figs. 4–10) H. and E. ×1800.

FIG. 4. Level of anterior portion of the esophageal bulb. The excretory cell is ventral to the esophagus.

FIGS. 5, 6. Levels of the posterior esophageal bulb and anterior intestine. The 2 excretory columns occupy much of the body cavity.

FIG. 7. Level just beyond the mid-intestine. The excretory columns are small structures in the lateral lines. An intestinal cell fills the body cavity.

FIGS. 8, 9. Levels of the intestine posterior to the excretory columns.

FIG. 10. Level posterior to the intestine.

PLATE I

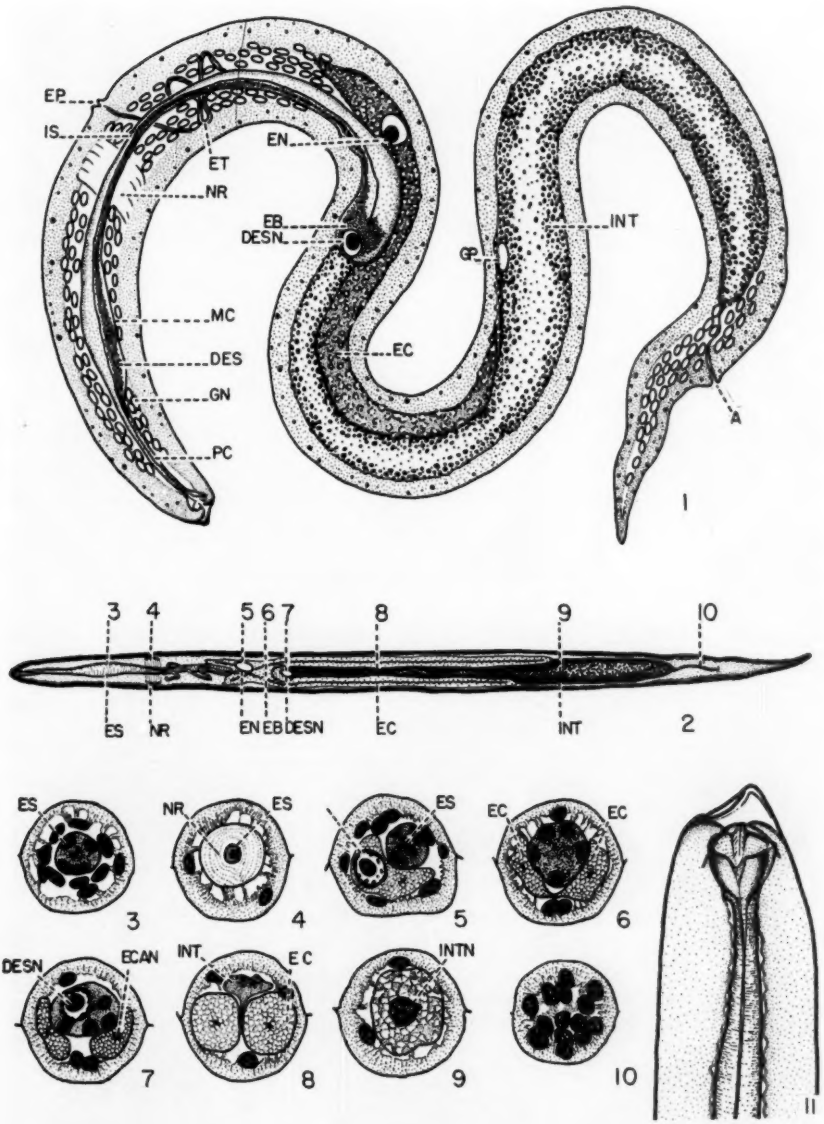
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PLATE II

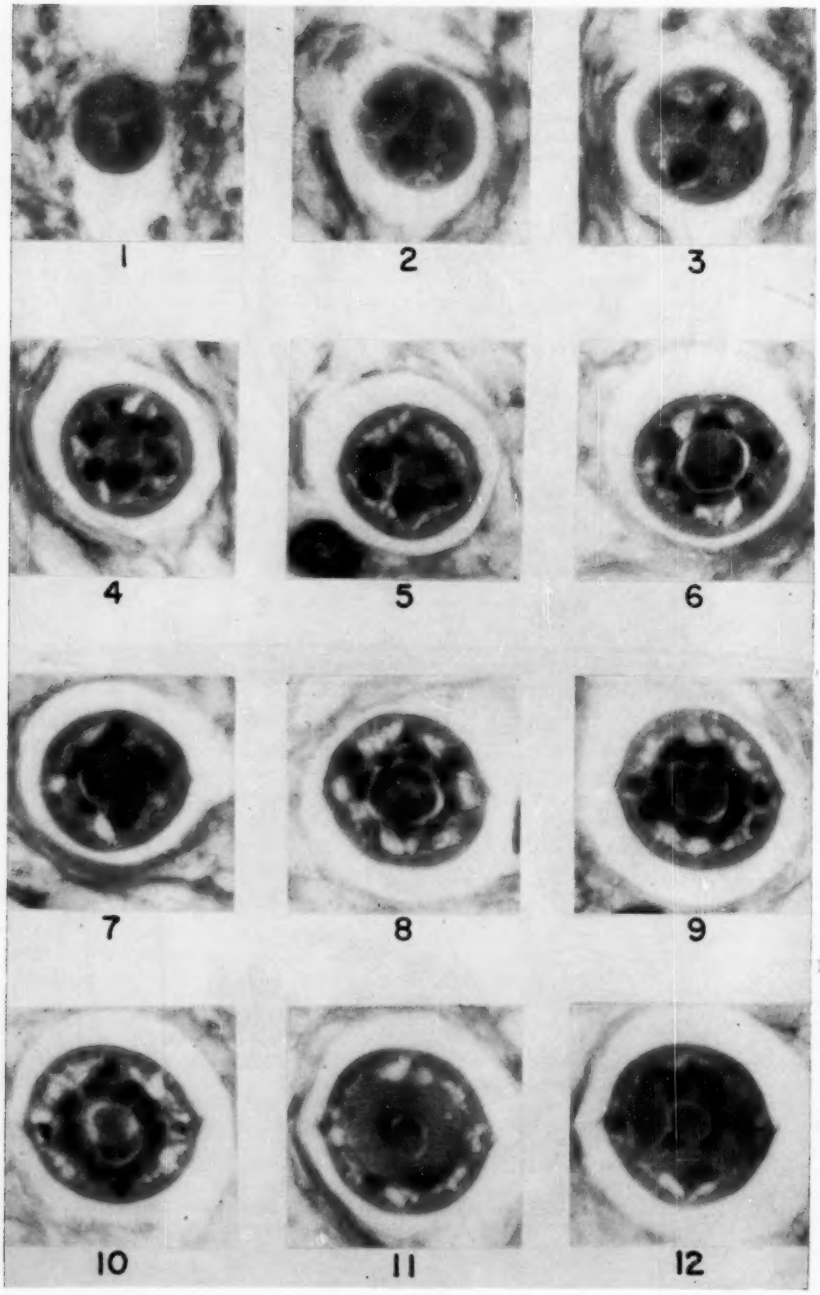


PLATE III

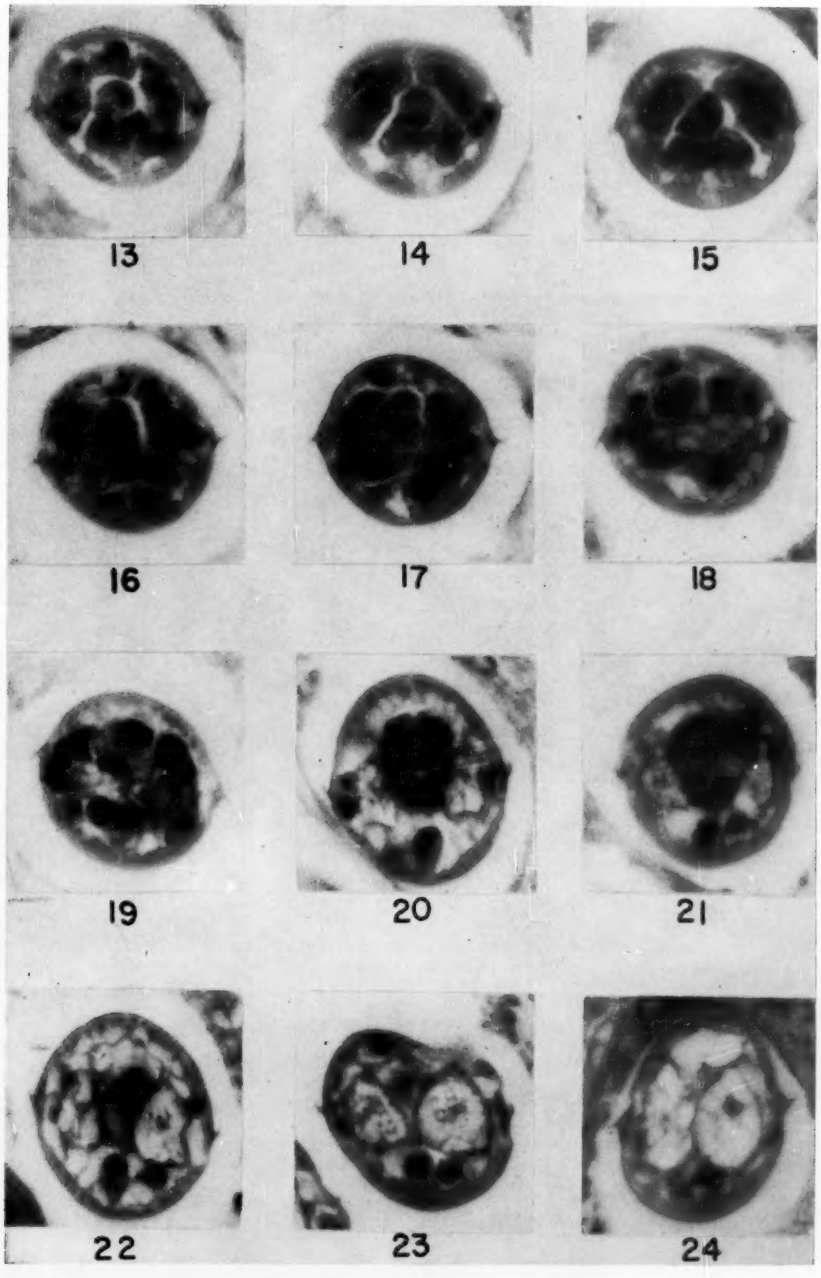
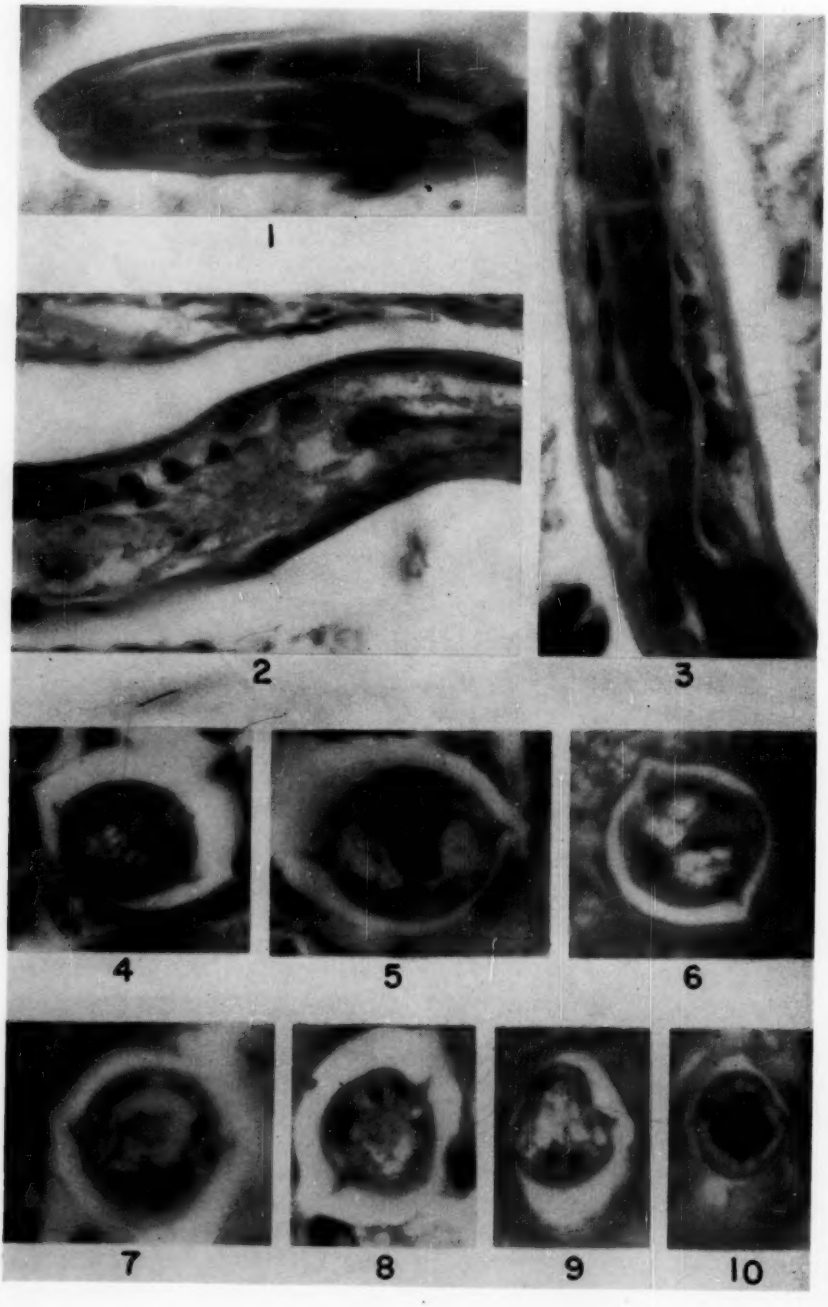


PLATE IV



THE ETIOLOGY OF VISCERAL LARVA MIGRANS

II. COMPARATIVE LARVAL MORPHOLOGY OF *ASCARIS LUMBRICOIDES, NECATOR AMERICANUS, STRONGYLOIDES STERCORALIS* AND *ANCYLOSTOMA CANINUM**

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Second-stage *Toxocara* larvae are recognized as etiologic agents of visceral larva migrans. It is strongly suspected that larvae of other species of nematodes may be capable of like behavior in the human body. In addition to those forms which have the potential ability to invade and survive for extended periods in human tissues, certain nematodes adapted to man have obligatory tissue phases in which the larvae follow a migratory route from the portal of entry to the trachea. Among the more common intestinal parasites which must be considered in the latter group are *Ascaris lumbricoides, Strongyloides stercoralis* and *Necator americanus.*

The migratory route of *Ascaris lumbricoides* was described by Ransom and Foster (1917, 1919, 1920), Ransom and Cram (1921) and Roberts (1934) who conclusively demonstrated that a liver-lung-trachea migration occurs in hogs, sheep and goats as well as in rats, mice, guinea pigs and rabbits. Proof that a similar migration takes place in man was provided by Koino (1922), who after ingesting 2000 embryonated eggs of human *Ascaris* developed pneumonia and produced hemorrhagic sputum containing numerous larvae. As regards the method of larval transport from the intestine to the liver and lungs, Ransom and Cram (1921) found the larvae to be passively carried by the blood and lymphatics, although they conceded the possibility of limited direct tissue penetration. Occasional larvae retained in lymph nodes of experimental animals developed to as much as 1.4 mm in length and were found 1 to 30 days after infection; larvae in the liver attained a length of 0.6 mm 4 to 6 days after infection. Growth to 2 mm occurred in the lungs although much smaller larvae occasionally were observed in tracheal migration. Larval growth patterns in experimentally infected rodents are probably essentially the same as those in human infection, since Roberts (1934) found the tissue phases of porcine *Ascaris* to be the same in both guinea pigs and hogs. The ability of many *Ascaris* larvae to enter the systemic circulation of experimental animals described by Ransom and Cram (1921), Sprent (1952) and others suggests that these larvae may invade many abnormal foci in human infection. Therefore, *Ascaris* larvae of various developmental stages may be encountered in the study of tissues from human biopsy and necropsy specimens and must be differentiated from *Toxocara.*

In general. *N. americanus* and *S. stercoralis* have similar migratory routes in human infection and may be encountered in the skin, subcutaneous tissues and lungs following percutaneous inoculation (Looss, 1911; Fülleborn, 1914, 1923).

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Schwartz and Alicata (1934) studied the developmental forms of *Necator* in experimentally infected guinea pigs. Third-stage larvae were recovered from the lungs following both percutaneous and oral inoculation. Molting forms (advanced third-stage) were observed in the lungs 4 to 8 days after infection. Similarly, growth of *Necator* in the lungs is believed to obtain in human infection (Lindquist, 1950). *Strongyloides* larvae have the ability to pass through the intact alveolar capillaries of experimental animals with resulting distribution by the systemic circulation (Fülleborn, 1914; Spindler, 1943). Systemic distribution has not been encountered with *Necator*. This difference in distribution may be explained in part by the minimal increase in size over that of the infective stage by larvae of the normal lung phase of *Strongyloides*. Moreover, *Strongyloides* larvae identical with the infective stage have the ability to remain in the tissues of the experimentally infected canine host for as long as 30 days (Faust, 1933). Evidence that *Strongyloides* larvae may be similarly retained in human skin was furnished by Faust and Kagy (1933). An important feature in strongyloidiasis, having no parallel in hookworm infection, is self-infection (Faust and DeGroat, 1940). Both autoinfection, resulting from soiling of the perianal region with feces and the subsequent penetration of infective larvae (Fülleborn, 1926), and internal autoinfection, brought about by the penetration of filariform larvae developing directly within the bowel are known to occur in experimentally infected animals and in human infections (Faust *et al.*, 1933, 1934; Faust, 1935). Tissue sections from a fatal case revealed larvae in the lower ileum, cecum, appendix, mesenteric lymph nodes and liver (Faust and DeGroat, 1940). In more recent reports of fatal internal autoinfection living third-stage larvae were found distributed in abnormal foci as above, especially the colon (Hartz, 1946, 1954).

The last nematode considered in this study, *Ancylostoma caninum*, is of interest for several reasons. *A. caninum* is a common hookworm of dogs and cats and has world-wide distribution. The infective third-stage larvae follow a typical skin-lung-trachea migration in the natural hosts (Fülleborn, 1914) and, furthermore, readily penetrate the skin of most warm blooded animals. Human contact with the infective forms certainly occurs with frequency, especially in regions having climatic and soil conditions conducive to the transmission of human hookworm. As regards the final disposition of the larvae in human tissue following inoculation, little is known. *A. caninum* adults only rarely have been recovered from the human intestine (Deane, 1950; Lie and Bras, 1950). Penetration of the human skin may cause little more than a transitory cutaneous eruption with restricted linear migration (White and Dove, 1928; Hunter and Worth, 1945). In experimentally infected rodents, *A. caninum* larvae produce an infection very similar to that caused by *Toxocara*. The larvae exhibit no growth or morphologic change (Scott, 1928; Nakajima, 1931) and can remain alive in the tissues for over 1 year (Matsusaki, 1950). In view of the relatively high incidence of *A. caninum* infection in dogs and cats, it appears very probable that the third-stage larvae of this species will be encountered in human tissues and that *A. caninum* may be an etiologic agent of visceral larva migrans.

METHODS

Ascaris lumbricoides eggs were obtained from gravid worms expelled from

children. The uterus was removed and placed in a 0.2% solution of sodium hypochlorite. The uterine tissue was dissolved within 2 hours, after which the free eggs were washed until clean. Incubation and inoculation into mice was conducted in the manner already described (Nichols, 1956). Infective filariform larvae of *N. americanus, A. caninum* and *S. stercoralis* were cultured and collected using previously described procedures (Beaver, 1953).

Animal infections were as follows: *A. caninum* in mice, rabbits, rats, hamsters and guinea pigs; *Strongyloides* in mice and rats; and *Necator* in mice only. Mice were separately inoculated by the oral route using a stomach catheter, and by the cutaneous route. The other animals were given percutaneous inoculations only. In most instances the numbers of larvae in the inocula were not accurately measured since the primary purpose was to introduce the greatest number compatible with a non-fatal pneumonitis. Fixation and staining of tissues and examinations of living and heat-killed larvae were conducted as previously described (Nichols, 1956). Digestion with pepsin was satisfactory only for *A. caninum* and advanced third-stage *A. lumbricoides;* the other larvae were destroyed by this technique. Those forms destroyed by digestion were collected by allowing the larvae to migrate from macerated tissues into physiological saline.

RESULTS

General Considerations

Development from the egg to the adult nematode includes 5 larval stages, each except the first and the fifth occupying a growth interval between successive molts, the fifth terminating in the sexually mature adult. In *Ascaris lumbricoides* the infective form is the second-stage larva within the egg. In *Strongyloides* and the hookworms it is the free third-stage filariform larva.

Ascaris lumbricoides. Tissue forms include all stages from the infective second to advanced third; the latter, when in the lungs, commonly begin tracheal migration. Second-stage larvae were recovered from various tissues as long as 2 weeks after infection, although the majority completed the third molt between 4 and 6 days. Molting larvae (advanced third) were found in the lungs from 8 to 14 days after infection. Living larvae were not recovered in the liver, lungs and carcass of 3 mice examined 3 weeks after infection and in 2 mice at 4 weeks. Dead larvae in the second, third, and fourth stages were found in the liver and lungs at both periods. Larvae from the kidneys and brain did not exceed the third stage. Only larvae in the lungs were able to complete growth to the fourth stage (*ca.* 2 mm long). Those in the liver and lymph nodes throughout the body reached a maximum length of 1.2 mm.

Necator americanus. Tissue forms include all stages from the infective third to the advanced third. The latter were found only in the lungs and trachea. Within 10 hours after either percutaneous or oral inoculation more larvae were found in the lungs than in any other organ. They could be recovered from the walls of the stomach and small intestine no later than 20 hours after oral inoculation. Muscles of the abdomen contained larvae until 48 hours after percutaneous inoculation. The liver was irregularly invaded up to 72 hours after oral inoculation; larvae were never recovered from this organ after percutaneous inoculation. Larvae were not found in the brain or kidneys. Molting larvae (advanced third)

were recovered from the lungs and trachea 5 to 12 days after infection, but at 15 and 17 days only dead larvae were recovered.

Strongyloides stercoralis. All observed tissue forms differed little in size from the infective third stage. Within one-half hour after either percutaneous or oral inoculation larvae in small numbers were found in the brain, lung, liver, kidney, spleen and heart blood. Maximal invasion of the lungs occurred 5 to 8 hours after inoculation; within 15 hours the majority of larvae had completed tracheal migration and were found in the stomach and intestinal contents. The kidney, spleen, brain and heart blood were consistently negative 12 hours after infection, although the somatic muscles, diaphragm, muscle layers of the stomach and small intestine, lungs and liver contained variable numbers of living larvae at 17 days. Only dead larvae and larval fragments were found in mice sacrificed 23, 24, 26 and 30 days after infection. Encapsulation was not observed.

Ancylostoma caninum. Larvae recovered from tissues, with few exceptions, included only the infective third stage. Several larvae which had undergone small increases in length and width were irregularly recovered in rat and mouse lung 4 to 8 days after infection. Forms of this size presumably underwent tracheal migration since all other larvae recovered from tissues at any interval were morphologically indistinguishable from the infective stage. Moreover, larvae from mouse lungs 3 and 7 days after infection and from brain at 9 days retained the ability to reinvade mice by both the oral and percutaneous routes. Resulting infections were typical, the larvae undergoing no increase in size. The migratory behavior and distribution of the larvae followed a similar pattern in the mouse, rat, guinea pig and rabbit. Percutaneous inoculation of infective larvae caused a pneumonitis in all animals, the severity and duration depending upon the number of larvae in the inoculum. Lung involvement was noted the first day after infection and persisted as long as 1 week. During this first week, larval invasion, especially of the central nervous system and somatic muscles, and to a lesser extent of the kidneys, liver, diaphragm, myocardium and salivary glands, was demonstrated in the mouse and rat. A single larva was recovered from the eye of 1 mouse at 8 days; the eyes were uniformly negative in all other animals, even in those mice having over 900 larvae in the brain. Invasion of tissues, other than the central nervous system, somatic muscles and lungs, occurred only irregularly except in animals given very heavy inocula. Larvae were never found in the myocardium, salivary glands, diaphragm and liver after the second week; the kidneys of all animals were negative after 30 days. Within the first 2 weeks of infection larval invasion of the brain and spinal cord in different animals varied widely. However, up to half of all larvae recovered from the entire body were found in the brain at 4 days after inoculation. Severe damage to the central nervous system resulting in death was incurred as early as the sixth day. Distribution of larvae to the somatic muscles was indicative of vascular dissemination; 30 or more days after infection larval counts of muscle samples from different widely separated areas were of similar magnitude. Living, actively motile larvae were recovered from the brain and somatic muscles of one mouse 392 days after inoculation, after which the study was terminated.

Morphology of Living and Heat-Killed Larvae

Ascaris lumbricoides. Second-stage larvae pressed from eggs are specifically

characterized by 3 lips which form a defined oral protuberance (Plate I, Fig. 1). These larvae are much smaller than *Toxocara,* a typical specimen having length and width of 260 and 14 microns respectively. All of the organ systems described for *Toxocara* are found in *Ascaris,* although their relationships are slightly different. The lateral alae are minute and extend to about 15 microns from anterior and posterior ends. The cuticle is without annulations. Ganglionic nuclei fill the body cavity obscuring much of the esophageal structure except the terminal bulb at the esophago-intestinal junction. The intestine is without lumen and consists of 7 cells packed with refractile granules. The single nucleus of the excretory cell is an oval structure about 6 microns in length and lies in a left ventro-lateral position of 15–20 microns anterior to the esophago-intestinal junction; little can be seen of excretory columns.

Larvae from 24-hour infections exhibit few changes (Table I). The esophagus is more robust and has a small enlargement just anterior to the nerve ring. The excretory cell nucleus is 8 microns in length and 2 posterior columns are found in the lateral lines extending 70–90 microns posterior to the esophago-intestinal junction.

At 48 hours, larvae from tissues show marked internal differentiation along with a slight increase in size (Table I; Plate II, Fig. 1). The ganglionic nuclei are grouped into dorsal, lateral and ventral bands and the esophageal structures are more defined Two enlarged portions 6–8 microns in diameter, the esophageal bulb and the section just anterior to the nerve ring, are connected by a narrow isthmus 4–5 microns in diameter.

Advanced second-stage larvae from 3- and 4-day infections have dimensions similar to those of infective *Toxocara* (Table I). The esophagus has uniform diameter, terminating in a bulb 13 microns wide (Plate II, Fig. 2). The nucleus of the esophageal gland in the dorsal field of the bulb is 3–4 microns in diameter and contains refractile granules similar to those in the excretory cell nucleus. The latter nucleus is at the level of the esophago-intestinal valve and has a maximum diameter of 12–14 microns. The posterior excretory columns extend to the genital primordium and the anterior portion of the cell forms 2 ventro-lateral columns which terminate midway between the cell nucleus and the excretory pore. Division of the intestinal cells is initiated in larvae 17–18 microns in width so that those with a width of 20 microns have 12 to 14 intestinal cells which form 2 rows bordering a narrow lumen.

Early third-stage larvae from 4- to 8-day infections have a range in length and width of 550–650 microns and 24–26 microns respectively (Table I). Labial papillae have appeared, 2 on the dorsal lip and 1 on each of the subventral lips. The esophagus and intestine are functional and particulate matter is present in the gut lumen. Nuclei of the 2 subventral esophageal glands can be distinguished and cell granules extend into the 3 fields of the esophagus. The nucleus of the excretory cell remains at the level of the esophago-intestinal valve and undergoes an enormous increase in size, frequently having a length equal to the diameter of the larvae. The excretory columns develop in a corresponding manner. Larvae 1 mm long have posterior columns extending the length of the intestine. Sexual dimorphism is first evident in the middle third stage, the sexes being distinguished by the caudal glands (Plate III, Figs. 15, 16).

Fourth-stage larvae, 1.7–2.0 mm in length differ from the advanced third stage in few respects; cuticular annulations, lacking in preceding stages, are present and the greatly enlarged posterior excretory columns together occupy a greater portion of the body cavity than does the intestine.

Necator americanus. The infective larva is enclosed in a larval sheath derived from the second-stage cuticle (Plate IV, Fig. 1). A typical specimen has length and width of 530 and 23 microns respectively (Table II). Double longitudinal alae extend in each lateral line to positions 30 microns from the extremities. The cuticle of the sheath and that of the body wall have heavy annulations. The oral apparatus consists of a buccal capsule 15 microns in depth and 3 microns in diameter at the widest portion. The heavy cuticular lining of the buccal capsule is directly continuous with the cuticle of the esophagus. In optical plane the heavy capsule wall appears as two "spears" about 12 microns in length. A small spherical vestibule without heavy walls is immediately anterior to the "spears" and forms the most anterior portion of the capsule. Young larvae have the intestinal cells and body wall filled with refractile granules. The body cavity of the esophageal

TABLE I.—*Mean dimensions, in microns, of* Ascaris lumbricoides *larvae pressed from the egg and recovered from mouse liver and lung at intervals ranging from 24 hours to 11 days after inoculation*

Duration of infection	Number of larvae	Source	Length		Width*		Esop.	Ex. pore	Anus
			Avg.	Range	Avg.	Range			
.....	30	egg	259	224–318	14	13–16	99	57	29
24 hours	20	liver	258	232–292	14	12–16	98	57	28
36 hours	14	liver	274	232–301	16	14–18	96	59	30
48 hours	15	liver	282	232–353	16	14–18	104	64	29
72 hours	10	liver	302	275–327	17	15–19	104	61	28
88 hours	10	liver	341	301–396	20	18–22	110	66	30
96 hours	5	liver	509	447–559	23	22–24	126	76	38
5 days	5	lung	564	516–602	28	28–30	142	87	44
6 days	5	lung	855	817–894	39	38–42	193	102	56
9 days	1	lung	1725	.....	54	.....	260	132	82
11 days	1	lung	1550	.....	50	.....	198	124	80

* Width, measured at esophago-intestinal junction, Esop., length from mouth to esophago-intestinal junction; Ex. pore, distance from mouth to excretory pore; Anus, distance from anus to tip of tail.

region is filled with ganglionic nuclei. The esophagus is a well formed organ with an irregularly outlined bulb having the posterior section divisible into 4 or more elements on the basis of esophageal gland granules. The esophagus has a diameter at the bulb of 15 microns, a 5-micron diameter at the level of the nerve ring and an enlargement 9 microns in diameter anterior to the nerve ring. The dorsal esophageal gland originates in the bulb and terminates shortly posterior to the nerve ring. The 2 subventral glands extend from the bulb to the esophageal enlargement anterior to the nerve ring. The excretory pore is found on the ventral line slightly posterior to the mid-esophageal level. The short excretory tube, about 15 microns in length, terminates in a pulsating ampulla. Two excretory cells, connected with the ampulla, form sacculate columns which are enlarged only at the anterior level. They extend in the lateral lines 40–50 microns posterior to the esophago-intestinal junction. The esophago-intestinal valve has 6 to 8 small nuclei and the intestine contains about 30 regularly spaced nuclei.

The first change noted in the growing third-stage larva is an increase in length of the excretory cells which can be traced to a level about 150 microns posterior to the esophago-intestinal valve (Plate IV, Fig. 2). Pulsatile activity of the

excretory ampulla is lost. Larvae from the lungs at 48 hours increase in length and width to 645 and 27 microns respectively (Table II). The intestinal lumen contains particulate matter in larvae 25–26 microns in diameter. The excretory cells continue to increase in size, especially at the level of the ampulla. Single nuclei appear in each lateral column as oval refractile bodies 18 microns in length. The right and left nuclei are 80 microns and 100 microns respectively posterior to the esophago-intestinal valve. Advanced third-stage larvae from the lungs

TABLE II.—*Mean dimensions, in microns, of* Necator americanus *larvae from charcoal cultures, and removed from mouse lung at intervals ranging from 48 hours to 6 days after infection*

Duration of infection	Number of larvae	Length		Width**		Esophagus	Ex. pore	Anus
		Avg.	Range	Avg.	Range			
.....	50*	535	499–576	23	22–24	164	98	62
48 hours	6	645	602–679	27	26–28	188	124	68
4 days	8	712	664–748	40	38–44	200	148	68
6 days	6	759	740–769	50	48–55	210	150	69

* Infective larvae from charcoal culture.
** Width, measured at esophago-intestinal junction; Esophagus, length from mouth to esophago-intestinal junction; Ex. pore, distance from mouth to excretory pore; Anus, distance from anus to tip of tail.

apparently lose the double lateral alae without undergoing a molt when a width of 30 microns is reached. The intestinal walls accumulate large amounts of pigment and compress the excretory columns into flat lateral bands. The third stage reaches a maximum width of 55 microns (Table II). In these forms the excretory columns extend 300 microns posterior to the esophago-intestinal valve and are without central canaliculi (Plate V, Figs. 1, 2). The latter structures are found only in the enlarged parts of the 2 cells near the ampulla. Three esophageal gland nuclei are conspicuous and lie at the same level in the bulb.

Strongyloides stercoralis. The infective larvae, about 500 microns long and 16 microns wide (Table III), are almost equally divisible into esophageal and intestinal regions (Plate VI, Fig. 1). Double lateral alae extend to levels about 40 microns from both extremities. The blunt oral structure shows little more than a round opening. The tail terminates with an irregular margin. The body cavity contains numerous ganglionic nuclei in the region of the nerve ring. The esophagus is slender throughout its length, consisting of little more than a nucleated sheath. At levels posterior to the nerve ring it is surrounded by 3 large esophageal glands, 1 dorsal, and 2 subventral, forming an integral unit which appears as a posterior esophageal enlargement. This enlargement has a width of about 12 microns, while the esophagus proper, anterior to the nerve ring, is about 6 microns wide. Refractile granules of the esophageal glands fill the posterior portions, the 2 subventral glands terminating a short distance anterior to the nerve ring and the dorsal gland continuing to the most anterior portion of the esophagus. At a level just anterior to the excretory pore the nerve ring forms an indistinct band of fibers encircling the esophagus. The excretory apparatus appears to consist of 2 oval nuclei, 8–10 microns in length, which lie in the subventral body cavity 15–30 microns posterior to the nerve ring, a terminal excretory tube 10 microns in length and a minute excretory pore in the ventral line at a level near the mid-esophagus. An esophago-intestinal valve containing 6 to 8 nuclei is interposed between the esophagus and the intestine which has 40 or more cells bordering on

Fourth-stage larvae, 1.7–2.0 mm in length differ from the advanced third stage in few respects; cuticular annulations, lacking in preceding stages, are present and the greatly enlarged posterior excretory columns together occupy a greater portion of the body cavity than does the intestine.

Necator americanus. The infective larva is enclosed in a larval sheath derived from the second-stage cuticle (Plate IV, Fig. 1). A typical specimen has length and width of 530 and 23 microns respectively (Table II). Double longitudinal alae extend in each lateral line to positions 30 microns from the extremities. The cuticle of the sheath and that of the body wall have heavy annulations. The oral apparatus consists of a buccal capsule 15 microns in depth and 3 microns in diameter at the widest portion. The heavy cuticular lining of the buccal capsule is directly continuous with the cuticle of the esophagus. In optical plane the heavy capsule wall appears as two "spears" about 12 microns in length. A small spherical vestibule without heavy walls is immediately anterior to the "spears" and forms the most anterior portion of the capsule. Young larvae have the intestinal cells and body wall filled with refractile granules. The body cavity of the esophageal

TABLE I.—*Mean dimensions, in microns, of* Ascaris lumbricoides *larvae pressed from the egg and recovered from mouse liver and lung at intervals ranging from 24 hours to 11 days after inoculation*

Duration of infection	Number of larvae	Source	Length		Width*		Esop.	Ex. pore	Anus
			Avg.	Range	Avg.	Range			
.....	30	egg	259	224–318	14	13–16	99	57	29
24 hours	20	liver	258	232–292	14	12–16	98	57	28
36 hours	14	liver	274	232–301	16	14–18	96	59	30
48 hours	15	liver	282	232–353	16	14–18	104	64	29
72 hours	10	liver	302	275–327	17	15–19	104	61	28
88 hours	10	liver	341	301–396	20	18–22	110	66	30
96 hours	5	liver	509	447–559	23	22–24	126	76	38
5 days	5	lung	564	516–602	28	28–30	142	87	44
6 days	5	lung	855	817–894	39	38–42	193	102	56
9 days	1	lung	1725	.....	54	.....	260	132	82
11 days	1	lung	1550	.....	50	.....	198	124	80

* Width, measured at esophago-intestinal junction, Esop., length from mouth to esophago-intestinal junction; Ex. pore, distance from mouth to excretory pore; Anus, distance from anus to tip of tail.

region is filled with ganglionic nuclei. The esophagus is a well formed organ with an irregularly outlined bulb having the posterior section divisible into 4 or more elements on the basis of esophageal gland granules. The esophagus has a diameter at the bulb of 15 microns, a 5-micron diameter at the level of the nerve ring and an enlargement 9 microns in diameter anterior to the nerve ring. The dorsal esophageal gland originates in the bulb and terminates shortly posterior to the nerve ring. The 2 subventral glands extend from the bulb to the esophageal enlargement anterior to the nerve ring. The excretory pore is found on the ventral line slightly posterior to the mid-esophageal level. The short excretory tube, about 15 microns in length, terminates in a pulsating ampulla. Two excretory cells, connected with the ampulla, form sacculate columns which are enlarged only at the anterior level. They extend in the lateral lines 40–50 microns posterior to the esophago-intestinal junction. The esophago-intestinal valve has 6 to 8 small nuclei and the intestine contains about 30 regularly spaced nuclei.

The first change noted in the growing third-stage larva is an increase in length of the excretory cells which can be traced to a level about 150 microns posterior to the esophago-intestinal valve (Plate IV, Fig. 2). Pulsatile activity of the

excretory ampulla is lost. Larvae from the lungs at 48 hours increase in length and width to 645 and 27 microns respectively (Table II). The intestinal lumen contains particulate matter in larvae 25–26 microns in diameter. The excretory cells continue to increase in size, especially at the level of the ampulla. Single nuclei appear in each lateral column as oval refractile bodies 18 microns in length. The right and left nuclei are 80 microns and 100 microns respectively posterior to the esophago-intestinal valve. Advanced third-stage larvae from the lungs

TABLE II.—*Mean dimensions, in microns, of* Necator americanus *larvae from charcoal cultures, and removed from mouse lung at intervals ranging from 48 hours to 6 days after infection*

Duration of infection	Number of larvae	Length		Width**		Esophagus	Ex. pore	Anus
		Avg.	Range	Avg.	Range			
.....	50*	535	499–576	23	22–24	164	98	62
48 hours	6	645	602–679	27	26–28	188	124	68
4 days	8	712	664–748	40	38–44	200	148	68
6 days	6	759	740–769	50	48–55	210	150	69

* Infective larvae from charcoal culture.
** Width, measured at esophago-intestinal junction; Esophagus, length from mouth to esophago-intestinal junction; Ex. pore, distance from mouth to excretory pore; Anus, distance from anus to tip of tail.

apparently lose the double lateral alae without undergoing a molt when a width of 30 microns is reached. The intestinal walls accumulate large amounts of pigment and compress the excretory columns into flat lateral bands. The third stage reaches a maximum width of 55 microns (Table II). In these forms the excretory columns extend 300 microns posterior to the esophago-intestinal valve and are without central canaliculi (Plate V, Figs. 1, 2). The latter structures are found only in the enlarged parts of the 2 cells near the ampulla. Three esophageal gland nuclei are conspicuous and lie at the same level in the bulb.

Strongyloides stercoralis. The infective larvae, about 500 microns long and 16 microns wide (Table III), are almost equally divisible into esophageal and intestinal regions (Plate VI, Fig. 1). Double lateral alae extend to levels about 40 microns from both extremities. The blunt oral structure shows little more than a round opening. The tail terminates with an irregular margin. The body cavity contains numerous ganglionic nuclei in the region of the nerve ring. The esophagus is slender throughout its length, consisting of little more than a nucleated sheath. At levels posterior to the nerve ring it is surrounded by 3 large esophageal glands, 1 dorsal, and 2 subventral, forming an integral unit which appears as a posterior esophageal enlargement. This enlargement has a width of about 12 microns, while the esophagus proper, anterior to the nerve ring, is about 6 microns wide. Refractile granules of the esophageal glands fill the posterior portions, the 2 subventral glands terminating a short distance anterior to the nerve ring and the dorsal gland continuing to the most anterior portion of the esophagus. At a level just anterior to the excretory pore the nerve ring forms an indistinct band of fibers encircling the esophagus. The excretory apparatus appears to consist of 2 oval nuclei, 8–10 microns in length, which lie in the subventral body cavity 15–30 microns posterior to the nerve ring, a terminal excretory tube 10 microns in length and a minute excretory pore in the ventral line at a level near the mid-esophagus. An esophago-intestinal valve containing 6 to 8 nuclei is interposed between the esophagus and the intestine which has 40 or more cells bordering on

a flattened lumen. The nuclei of the latter structure are regularly arranged in 2 columns of cells. The genital primordium appears as a cellular mass 28–30 microns in length and is located in the ventral line 80–90 microns posterior to the esophagointestinal valve.

Ancylostoma caninum The infective larva is larger but has a shape similar to that of *Necator* (Plate VII). A typical specimen has length and width of about 660 and 22 microns respectively (Table III). Double longitudinal alae extend in each lateral line to positions 30–40 microns from the extremities. The cuticle has only faint circular annulations. The oral apparatus consists of a buccal capsule about 15 microns in depth and less than 3 microns in diameter at the widest portion. Heavy cuticular walls line the capsule and are directly continuous with the cuticular lining of the esophagus. In optical plane the capsule walls appear as 2 "spears" 12 microns long, having their anterior ends directed toward the opposite member while having no convexities as does *Necator*. A short channel of narrow caliber and without heavy walls is located just anterior to the "spears" and forms the most external portion of the capsule. The body cavity is filled with ganglionic nuclei which are especially numerous posterior to the nerve ring. The esophagus extends as a column of almost uniform diameter from a level 16 microns posterior to the oral opening to the enlarged posterior bulb. The column has a

TABLE III.—*Mean dimensions, in microns, of infective third-stage larvae of* Strongyloides stercoralis *and* Ancylostoma caninum *from charcoal cultures*

Larvae species and number	Length		Width*		Esophagus	Ex. pore	Anus
	Avg.	Range	Avg.	Range			
S. str. 35	505	456–568	16	15–16	235	103	64
A. can. 30	664	576–697	22	20–24	170	124	76

* Width, measured at esophago-intestinal junction; Esophagus, length from mouth to esophago-intestinal junction; Ex. pore, distance from mouth to excretory pore; Anus, distance from anus to tip of tail.

diameter of 6–7 microns from its most anterior extension to a level just anterior to the nerve ring, where it forms a slight enlargement 8 microns wide. Posterior to the nerve ring at the level of the excretory ampulla the esophagus begins to increase in diameter, reaching a maximum of 14–15 microns at the bulb. The dorsal esophageal gland extends as a column of granules the entire length of the esophagus from the bulb to the buccal capsule. The 2 subventral glands originate in the bulb and terminate in the esophageal enlargement anterior to the nerve ring, just anterior to the excretory pore. The excretory pore in the mid-ventral line is found at the beginning of posterior third of the esophagus. A short tube 12–15 microns in length, the terminal excretory tube, connects the excretory pore with a pulsating ampulla which maintains continuous but irregular activity. The excretory pore opens and closes in synchronization with pulsations of the ampulla. Two excretory cells form long sacculate columns which extend in the lateral line from the ampulla to the posterior intestine, the left column terminating near the end of the intestine and the right column terminating 60–80 microns posterior to the genital primordium. The nucleus of the left cell lies in the body cavity 100 microns posterior to the esophageal bulb; that of the right cell is about 30 microns nearer the bulb. The nuclei are oval bodies having a maximum length and width of 17 and

8 microns respectively. The esophago-intestinal valve contains 6–8 nuclei of smaller size than those of the esophagus. The intestine is composed of about 30 cells with regularly arranged nuclei. The genital primordium, an oval mass of about 16 cells, is located in the ventral line firmly adherent to the intestine, 180–200 microns anterior to the anus.

Interpretation of Tissue Sections

Ascaris lumbricoides, second-stage larva (diameter, 13–16 microns). Longitudinal sections of the esophageal region show little more than a mass of basophilic ganglionic nuclei (Plate IX, Fig. 1). At the appropriate levels, however, the nerve ring and the characteristic excretory cell nucleus are evident (Plate I, Fig.2). Sagittal sections of the mid-gut region show 7 regularly spaced intestinal cell nuclei (Plate I, Fig. 2; Plate IX, Fig. 7). The numerous ventral-line nuclei form an almost unbroken column from the esophagus to the anus (Plate I, Fig. 2). The cuticle, hypodermis, muscle layer and intestinal-cell matrix are acidophilic, each staining similarly with eosin. The esophagus anterior to the excretory cell nucleus is a thin tube commonly having 2 nuclei in cross section (Plate I, Fig. 3); at the bulb 4 to 6 nuclei are frequently seen (Plate I, Fig. 8; Plate IX, Fig. 3). Minute lateral alae commence at a level just anterior to the nerve ring and extend to the level of the anus (Plate I, Figs. 4–11; Plate IX, Figs. 4, 5). The nucleus of the excretory cell contains 1 or more large basophilic masses and numerous small granules of uniform size (Plate I, Figs. 2, 6, 7; Plate IX, Fig. 3). There is no evidence of acidophilic cytoplasm associated with the excretory cell at any level. In transverse sections of the mid-gut, the intestinal cells appear as a faint eosinophilic matrix (Plate I, Fig. 9; Plate IX, Figs. 4, 5, 8), unless the section is at a level of one of the 7 nuclei (Plate I, Fig. 10). Transverse sections posterior to the intestine show only numerous basophilic nuclei filling the body cavity (Plate I, Fig. 11).

Advanced Second Stage (maximum diameter, 23 microns). Nuclei are absent in the blunt anterior portion of the body, both in the esophagus and the body wall (Plate III, Fig. 1). The nucleus of the dorsal esophageal gland has an internal structure similar to that of the excretory cell nucleus but has a diameter of only 5 microns, about half of that of the esophageal bulb (Plate III, Figs. 1, 4; Plate X, Fig. 6). The intestine now consists of 12 to more than 20 cells (Plate III, Fig. 1; Plate XI, Fig. 1). However, neither it nor the esophagus has a patent lumen. The ventral line contains many nuclei; the dorsal line has fewer. The tail is still relatively undifferentiated (Plate III, Fig. 1; Plate XI, Fig. 2). Transverse sections of the esophagus show a well formed organ divisible into 1 dorsal and 2 subventral fields; 4 or 5 nuclei may be seen (Plate III, Figs. 2, 3; Plate X, Fig. 5). The bulb may have as many as 8 nuclei in a section (Plate III, Fig. 4; Plate X, Fig. 6). The nucleus of the excretory cell is associated with a small amount of acidophilic cytoplasm and 2 lateral excretory columns extend into the mid-intestinal region (Plate III, Figs. 5–7; Plate X, Fig. 7). The maximum diameter of the columns does not exceed 6 microns, approximately one-quarter of the body diameter. The columns are uniformly eosinophilic and have central canaliculi posterior to the esophago-intestinal junction. Lateral alae are inconspicuous and confined to levels between the nerve ring and posterior intestine (Plate III, Figs. 3–7; Plate X, Figs. 3–7).

Early Third Stage (maximum diameter, 32 microns). These larvae resemble the advanced second stage in general structure. The lateral alae remain relatively inconspicuous (Plate XI, Figs. 3, 5), and the excretory cell, although undergoing considerable development, retains the same general size relationship (Plate XI, Figs. 4, 5); the posterior columns remain smaller in diameter than the intestine. However, the esophagus and intestine become functional when the larvae reach a diameter of 24–26 microns and consequently particulate matter is found in the gut.

Advanced Third Stage (maximum diameter, 55 microns). These larvae are characterized by strong, well developed lateral alae and by a comparatively enormous development of the excretory cell (Plate III, Figs. 10, 14; Plate XII, Figs. 2, 4). Transverse sections at the mid-intestinal level show large posterior columns, the left column always having a greater diameter than the right (Plate III, Figs. 8, 11; Plate XII, Fig. 3). The nucleus of the excretory cell has so increased in size that its length is equal to the diameter of the larva at the esophago-intestinal junction (Plate III, Fig. 14; Plate XII, Fig. 4). The esophagus has an almost uniform diameter throughout its length with a reduced size at the level of the nerve ring (Plate III, Fig 9). The nuclei of the 2 subventral esophageal glands are at nearly the same level as the larger nucleus of the dorsal gland (Plate III, Figs. 10, 14). The intestine may have 4 or more nuclei in transverse section and commonly has particulate matter within the lumen (Plate XII, Figs. 1, 3).

Necator americanus, third-stage larva (maximum diameter, 28 microns). The esophagus is well demonstrated in sagittal section even though ganglionic nuclei fill the body cavity in both directions from the nerve ring. Esophageal nuclei are round to oval in the bulb and elongate in levels anterior to the nerve ring; they are absent from the region just posterior to the nerve ring extending halfway to the esophago-intestinal valve. Nuclei of the bulb have similar morphology and staining properties as in the earliest forms; the 3 esophageal gland nuclei cannot be distinguished from the muscle nuclei. The esophago-intestinal valve nuclei are invaginated into the lumen of the gut, the nuclei appearing as closely associated pairs. The intestine is round and tubular, and frequently is the only structure seen within the body cavity in either transverse or longitudinal sections (Plate IV, Figs. 7–9; Plate XIII, Figs. 3, 4). Small excretory columns are seen infrequently. Particulate matter is found in the intestinal lumen of larvae 26–28 microns wide and a well defined triradiate lumen is seen in transverse sections of the esophagus (Plate IV, Figs. 4–8). The esophageal gland nuclei can be differentiated from muscle nuclei in larvae of this size (Plate IV, Fig. 6). The former have about twice the diameter of other esophageal nuclei and each contains a single large basophilic granule. The 2 excretory cell nuclei have increased in size and are associated with small flattened posterior columns (Plate IV, Figs. 7, 8; Plate XIII, Fig. 3). These 2 cells are most evident, however, at levels between the nerve ring and the esophageal bulb. Double lateral alae are found in transverse sections and are especially evident through the gut and posterior esophageal levels (Plate IV, Figs. 4–9; Plate XIII, Figs. 2, 4).

Advanced Third Stage (maximum diameter, 50 microns). Lateral alae are no longer evident on larvae 30 to 50 microns in diameter. The 3 esophageal glands fill the esophagus from the bulb to the nerve ring and appear in transverse sections as vacuolated areas which separate the radial muscle fibers (Plate V, Figs. 8–11;

Plate XIII, Fig. 5). The 2 excretory cells have a greatly increased diameter between the esophageal bulb and nerve ring, where they may appear either as a united body or as 2 separate structures (Plate V, Figs. 3, 9–11; Plate XIII, Figs. 5, 6). The posterior extensions appear as flattened columns in the lateral lines (Plate V, Fig. 13). The intestine appears to be composed of the same number of cells as in the infective larva, no more than 2 cells being found in transverse sections (Plate V, Figs. 12–14). The individual cells have increased in size to such an extent that they completely fill the body cavity (Plate XIII, Fig. 7; Plate XIV, Fig. 1).

Strongyloides stercoralis (maximum diameter, 16 microns). The enlarged posterior esophageal region, consisting in large part of the 3 esophageal glands, stains intensely with hematoxylin and frequently obscures all structural details (Plate XVI, Fig. 3). The esophagus anterior to the nerve ring is highly vacuolated and has less affinity for stain (Plate XVI, Fig. 1). In median sagittal sections the central esophageal tube may be seen as a slender uniform column extending from the esophago-intestinal valve to the nerve ring (Plate VI, Fig. 2). In transverse sections the esophagus is rarely recognizable posterior to the nerve ring (Plate VI, Figs. 7–10; Plate XVIII, Figs. 2–4). Anterior to the nerve ring the esophagus is more robust and is not associated with glandular portions of the esophageal glands (Plate VI, Fig. 3). In transverse sections immediately anterior to the nerve ring the esophagus has a prominent triradiate lumen. Tubules, possibly of esophageal gland origin, appear in the periphery interposed between muscle nuclei of the esophagus (Plate VI, Fig. 5; Plate XVI, Fig. 4). At more anterior levels these structures are less pronounced and little more than esophageal nuclei are seen (Plate VI, Fig. 4; Plate XVI, Fig. 5). Three large gland nuclei can be seen in longitudinal and transverse sections 5–25 microns anterior to the esophago-intestinal junction (Plate VI, Figs. 2, 10; Plate XVII, Figs. 1, 3). These nuclei are uniformly oval and each contains a single large basophilic granule. Ganglionic nuclei are numerous only at levels close to the nerve ring (Plate VI, Figs. 6, 7; Plate XVII, Fig. 2). The intestine consists of a double row of closely opposed cells with nuclei having about twice the diameter of those of the body wall (Plate VI, Fig. 2; Plate XVI, Fig. 2). In transverse section 1 or 2 intestinal cell nuclei may be seen in an homogeneous eosinophilic mass without evidence of a central lumen (Plate VI, Figs. 11, 12; Plate XVII, Fig. 5). The genital primordium appears as a basophilic mass closely adherent to the wall of the intestine (Plate XVI, Fig. 2). Muscle and hypodermal nuclei of the body wall are difficult to distinguish at all levels. Double lateral alae are conspicuous at most levels from the nerve ring to the anus (Plate VI, Figs. 5–13; Plate XVII, Figs. 4–6). Frequently, however, the alae and associated cuticle are widely separated from the body wall, so that they lose their characteristic form. Material from 2 human infections that were reported by Dr. Philip H. Hartz (1954) and generously supplied by him, enabled comparison between *Strongyloides* morphology in the abnormal host and in human tissues. The larvae proved to be structurally identical and the preceding description has been based upon *Strongyloides* from both sources.

Ancyclostoma caninum (maximum diameter, 24 microns). The esophagus resembles that of the early tissue phases of *Necator* (Plate VIII, Fig. 1). At levels 20–30 microns posterior to the buccal capsule it fills the body cavity and

contains 3 uniformly basophilic muscle nuclei, 1 in each field (Plate VIII, Fig. 3; Plate XV, Fig. 2). The nucleus in the dorsal field commonly is associated with a small vacuole which may represent the anterior extension of the dorsal esophageal gland. Nearer the nerve ring 4 to 6 nuclei are found in transverse sections along with a vacuole in the dorsal field (Plate VIII, Figs. 4, 5). At the nerve ring, only 3 esophageal nuclei are seen and each is associated with a vacuole. The glandular portion of the esophagus, posterior to the nerve ring, is permeated with numerous inter-connecting channels which appear in transverse section as small irregular vacuoles (Plate VIII, Fig. 6; Plate XV, Fig. 3). Sections through the bulb fail to disclose gland nuclei (Plate VIII, Fig. 7). In longitudinal sections the intestine is seen as a multi-nucleated organ only at levels 35–40 microns posterior to the esophageal bulb and at its most caudal extension (Plate VIII, Fig. 1). The central portion of the intestine is strongly compressed by posterior extensions of the 2 excretory cells. The nuclei of the 2 excretory cells are uniformly basophilic and show no internal structure (Plate VIII, Figs. 1, 8; Plate XV, Figs. 1, 5). They form an important diagnostic feature since sections at these levels show little more than 2 large nuclei. The genital primordium frequently appears very similar to the excretory cell nuclei (Plate XIV, Fig. 3). The posterior columns of the excretory cells are filled with a light but uniformly staining matrix; central excretory canaliculi are not evident (Plate VIII, Figs. 8–10; Plate XV, Figs. 4–6). At levels extending from just anterior to the excretory cell nuclei posteriorly to slightly beyond the genital primordium the excretory columns occupy the major portion of the body cavity. Both columns may be of nearly equal size or one may greatly exceed the other. The caliber of the intestinal lumen markedly increases posterior to the genital primordium where the excretory columns are of small size (Plate VIII, Figs. 10, 11; Plate XV, Fig. 7). Prominent double lateral alae are found at all levels from the nerve ring to the anus (Plate VIII, Figs. 3–14; Plate XV, Figs. 2–9).

DISCUSSION

From the morphologic descriptions presented here and in a previous report (Nichols, 1956) it is evident that specific differentiation of nematode larvae in stained tissue sections frequently requires attention to relatively few structural features. Measurements of absolute length and width have limited value, since all of the larvae exhibit some variation and have tissue phases with diameters falling within the range of about 15 to 22 microns. Furthermore, measurements of length frequently entail laborious serial reconstruction and are therefore subject to large error. On the other hand, a single good transverse section of mid-intestinal levels may furnish sufficient material for specific recognition. Diagnosis at this level is based upon the relative size of the larva, type of intestine, presence and type or lack of lateral alae and the presence and size or lack of excretory columns. The following diagnostic key based upon these 4 characters is applicable to the 6 species of larvae thus far described.

1. Diameter at mid-gut, 14–16 microns; lateral alae prominent 2
   Diameter at mid-gut, greater than 26 microns; lateral alae present or absent 5
   Diameters at mid-gut, 14–17 microns; lateral alae minute and single ..... early second-stage *Ascaris lumbricoides*
2. Lateral alae single 3
   Lateral alae double 6

3. Single gut cell; posterior excretory columns large, well defined; minute central excretory canaliculi frequently present 4
Intestine with prominent lumen and composed of 2 or 3 cells; excretory columns small, well defined .....
advanced second-stage *Ascaris lumbricoides*

4. Diameter at mid-gut, 16 microns or less .....
early second-stage *Toxocara cati*
Diameter at mid-gut, 18–22 microns .....
early second-stage *Toxocara canis*

5. Diameter at mid-gut, 26–50 microns .....
Lateral alae absent; excretory columns reduced or absent .....
advanced third-stage *Necator americanus*
Lateral alae prominent; excretory columns with cross-section area equal to or greater than intestine .....
second to third-stage *Ascaris lumbricoides*

6. Diameter at mid-gut, 22–24 microns; excretory columns large, well defined; central excretory canaliculi lacking .....
early third-stage *Ancylostoma caninum*
Diameter at mid-gut, 14–26 microns; excretory columns reduced or lacking 7

7. Diameter at mid-gut, 22–26 microns; 2 gut cells filling the body cavity .....
early third-stage *Necator americanus*
Diameter at mid-gut, 14–16 microns; 2 gut cells not filling the body cavity .....
early third-stage *Strongyloides stercoralis*

Transverse sections of the esophageal region are more difficult to interpret. However, since the lateral alae are found in this region as well as at posterior levels, larvae may be recognized as belonging to the hookworm, ascarid or *Strongyloides* group on the basis of the body diameter and the lateral alae. Further differentiation depends largely upon the character of the esophagus, the presence or absence of a single excretory cell nucleus and the esophageal gland nuclei. Therefore, specific diagnosis can be made only infrequently with single transverse sections at the esophageal level.

In longitudinal section the second-stage ascarid larvae are difficult to diagnose since the lateral alae cannot be observed and the true width of the body can only be estimated. The numerous ganglionic nuclei in the body cavity of the esophageal region tend to mask the esophagus and excretory apparatus, so that only median sagittal sections, no more than 5 microns in thickness, will clearly show the internal structures. Diagnosis is then based on the character of the esophagus and the size and relationship of the excretory cell nucleus to the esophago-intestinal junction. The excretory cell of *Ascaris* has a maximum length equal to at least one-half the diameter of the body and is commonly found no more than 10 microns anterior to the esophago-intestinal junction. That of *Toxocara* larvae has a maximum length equal to about one-third the body diameter and is found 20–25 microns anterior to the esophago-intestinal junction. Longitudinal sections of the intestinal region of *Ascaris* show only the intestinal cells whereas in similar sections of *Toxocara* the intestine is only infrequently seen since the 2 posterior excretory columns occupy almost all of the body cavity at levels from gut cells 1 to 4.

Although early third-stage *Ancylostoma caninum* and *Necator* cannot be separately distinguished at esophageal levels, they are readily differentiated from the ascarids. A relative lack of ganglionic nuclei in longitudinal sections and vacuolated channels in the esophagus in transverse sections are characteristic of the hookworms and are the most specific characters available at levels near and anterior to the nerve ring. Occasional transverse sections at the mid-gut level of *A. caninum* larvae bear a striking resemblance to mid-gut levels of second-stage

Toxocara. Posterior excretory columns fill the body cavity of both forms and the intestine may be compressed into an unrecognizable form. If the lateral alae are not present, due to loss or separation of the cuticle or to a tangential section, attention to other characters is necessary. The excretory columns of *Toxocara* have a regular, round shape and each contains a minute central canaliculus; the intestine is compressed dorsad and is usually not recognized unless the section bisects 1 of the 7 nuclei. *A. caninum*, on the other hand, has columns of more irregular shape, with one column frequently much larger than the other and they lack central canaliculi. The intestine is a delicate structure often with collapsed walls compressed in the central body cavity; 1 or 2 intestinal cell nuclei are almost always seen.

Strongyloides larvae are readily recognized and cannot be confused with the other forms of similar diameter. The muscle and hypodermal nuclei are not much larger than bacteria and structures of the body wall are well differentiated. The muscle cells, 2 in each quadrant, and the hypodermis frequently appear as separate layers. Transverse and longitudinal sections of the esophageal region show few ganglionic nuclei, most of which are limited to the ventral line and to areas near the nerve ring. The entire esophagus is basophilic and the muscle nuclei are poorly differentiated. The excretory system is most difficult to find and does not have a size at all comparable to the large structures of ascarid and hookworm larvae.

The behavior of *A. caninum* and *Toxocara* in the abnormal rodent host is similar in that larvae of both species are capable of existing in different tissues for periods well in excess of 1 year. If *A. caninum* and possibly other larvae behaving in a similar manner in the abnormal rodent host are capable of like behavior in man, they might be expected to produce a disease syndrome characterized by transitory pneumonitis, prolonged eosinophilia and symptoms referable to central nervous system injury. Visceral larva migrans with *Toxocara* is confined largely to young children of the dirt-eating age. On the other hand, skin-penetrating larvae have more frequent contact with older children and adults, especially in rural areas.

Tropical eosinophilia is characterized by chronic pneumonitis, prolonged eosinophilia and therapeutic response to arsenicals (Ball, 1950; Danaraj, 1951; Aitken and Roy, 1953). The disease usually appears during the rainy season in rural tropical and sub-tropical regions, and all age groups of both sexes are subject to infection. Different agents have been suggested as the etiologic factor (certain nematodes, spirochetes, mites and viruses), although none appear to have adequate relationship to the disease process. The findings of previous workers and those of the present study, together with the known distribution and epidemiology of the disease, strongly suggest a larval nematode etiology. Furthermore, the occurrence of the disease in all age groups, the increased incidence during the rainy season and the infrequently associated hepatic involvement point toward a skin-penetrating species.

SUMMARY

The comparative morphology of the tissue stages of *Ascaris lumbricoides, Necator americanus, Strongyloides stercoralis* and *Ancylostoma caninum* is described. These larvae can most easily be distinguished from each other and from second-stage *Toxocara* by features seen in transverse sections at the level of the mid-intestine. Specific diagnosis of all forms is based on the presence and size

or lack of lateral alae and posterior excretory columns, the type of intestine and the relative diameter of the body.

Although *A. lumbricoides*, *Strongyloides* and *N. americanus* larvae invade many different tissues during their normal migratory phase and are potentially capable of entering the systemic circulation, their behavior in experimentally infected abnormal hosts suggests that they are incapable of prolonged existence in human tissue.

Similarities in behavior exhibited by *T. canis* and *A. caninum* larvae suggest the latter is also capable of producing visceral larva migrans in man. It is suggested that skin-penetrating larvae of the *A. caninum* type may be involved in the etiology of tropical eosinophilia.

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EXPLANATION OF PLATES

PLATE I

All drawings were made with the aid of a camera lucida.

FIG. 1. Second-stage *Ascaris lumbricoides* larva pressed from the egg. Heat-killed, unstained. ×870.

FIGS. 2–11. Early second-stage *A. lumbricoides* 24 hours in mouse liver. H. and E. Fig. 2, × 870; Figs. 3–11, × 1600.

FIG. 2. Reconstruction of an entire larva.
FIG. 3. Level anterior to nerve ring.
FIG. 4. Level of nerve ring.
FIG. 5. Level posterior to nerve ring.
FIG. 6. Level of excretory nucleus.
FIG. 7. Level 5 microns posterior to Fig. 6.
FIG. 8. Level of esophageal bulb.
FIG. 9. Level of mid-intestine.
FIG. 10. Level of posterior intestine transecting an intestinal cell nucleus.
FIG. 11. Level posterior to intestine.

A, anus; AMP, excretory ampulla; DES, dorsal esophageal gland; NDES, nucleus of dorsal esophageal gland; EB, esophageal bulb; EC, excretory columns; EN, nucleus of excretory cell; EP, excretory pore; ES, esophagus; EV, esophago-intestinal valve; GN, ganglionic nucleus; GP, genital primordium; HN, hypodermal nucleus; INT, intestine; INTN, nucleus of intestinal cell; NR, nerve ring; RGN, rectal gland nucleus; SMN, somatic muscle nucleus; VES, ventral esophageal gland; VESN, nucleus of ventral esophageal gland.

PLATE II

FIG. 1. *Ascaris lumbricoides*. Camera lucida drawing of an early second-stage larva 48 hours in mouse liver. Heat-killed, unstained. ×870.

FIG. 2. *A. lumbricoides*. Camera lucida drawing of an advanced second-stage larva 3 days in mouse liver. Heat-killed, unstained. × 650.

PLATE III

All drawings were made with the aid of a camera lucida.

Advanced second-stage *Ascaris lumbricoides* larvae 88 hours in mouse liver. H. and E. Fig. 1, ×730; Figs. 2–7, ×1000.

FIG. 1. Reconstruction of an entire larva.
FIG. 2. Level anterior to nerve ring.
FIG. 3. Level of nerve ring.
FIG. 4. Level of posterior esophageal bulb.

FIG. 5. Level of mid-intestine.
FIG. 6. Level of esophago-intestinal valve.
FIG. 7. Level of anterior intestine.
Advanced third-stage *A. lumbricoides* larvae 8 days in mouse lung.
(FIGS. 8–14). H. and E. ×560.
FIG. 8. Level of mid-intestine. Formalin fixation.
FIG. 9. Level just posterior to nerve ring.
FIG. 10. Level of excretory cell nucleus.
FIG. 11. Level of mid-intestine. Bouin fixation.
FIG. 12. Rectal gland nucleus of female larva.
FIG. 13. Rectal gland nucleus of male larva.
Advanced third-stage *A. lumbricoides* larvae 10 days in mouse lung.
FIG. 15, male; FIG. 16, female. Heat-killed, unstained. ×430.

PLATE IV

All drawings were made with the aid of a camera lucida.
FIG. 1. Infective third-stage *Necator americanus*. Heat-killed, unstained. ×430.
FIG. 2. *N. americanus*. Dorsal aspect of larva 48 hours in mouse lung. Heat-killed, unstained. ×870.
Early third-stage *N. americanus* 48 hours in mouse lung (Figs. 3–9). H. and E. ×560.
FIG. 3. Level 20 microns anterior to nerve ring.
FIG. 4. Level of nerve ring.
FIG. 5. Level 5 microns posterior to nerve ring.
FIG. 6. Level of posterior esophageal bulb.
FIG. 7. Level of anterior intestine.
FIG. 8. Level of one excretory nucleus.
FIG. 9. Level of posterior intestine.

PLATE V

All drawings were made with the aid of a camera lucida.
FIG. 1. Advanced third-stage *Necator americanus* 4 days in mouse lung. Ventral view. Heat-killed, unstained. ×430.
FIG. 2. Buccal structure of advanced third-stage *Necator*. Heat-killed, unstained. ×430.
Advanced third-stage *Necator* 5 days in mouse lung (Figs. 3–16). H. and E. ×560.
FIG. 3. Median sagittal section.
FIGS. 4–6. Successive levels anterior to nerve ring.
FIGS. 7–11. Successive levels from nerve ring to posterior esophageal bulb.
FIG. 12. Level of mid-intestine.
FIG. 13. Level near posterior termination of excretory columns.
FIG. 14. Level posterior to excretory columns.
FIG. 15. Level midway between intestinal termination and anus.
FIG. 16. Level posterior to anus.

PLATE VI

All drawings were made with the aid of a camera lucida.
FIG. 1. *Strongyloides stercoralis* larva after 12 days in rat abdominal muscle. Heat-killed, unstained. ×730.
S. stercoralis larva in lamina propria of human colon (Hartz material) H. and E. Figs. 2, 3, ×780; Figs. 4–13, ×1200.
FIG. 2. Posterior esophageal region.
FIG. 3. Esophageal region anterior to nerve ring.
FIGS. 4, 5. Two levels anterior to nerve ring.
FIGS. 6–10. Successive levels from nerve ring to posterior esophagus.
FIG. 11. Intestinal level showing one intestinal cell.
FIG. 12. Intestinal level showing two intestinal cells.
FIG. 13. Level posterior to anus.

PLATE VII

Camera lucida drawing of an infective third-stage *Ancylostoma caninum*. Heat-killed, unstained. ×870.

PLATE VIII

Camera lucida drawings of third-stage *Ancylostoma caninum* 30 days in mouse brain. H. and E. Fig. 1, ×430; Figs. 2–14, ×1500.

FIGS. 2–5. Successive levels from buccal capsule to nerve ring.

FIG. 6. Level of glandular esophagus.

FIG. 7. Level of posterior esophageal bulb.

FIG. 8. Level of an excretory cell nucleus.

FIG. 9. Level of mid-intestine.

FIG. 10. Level near termination of excretory columns.

FIG. 11. Level posterior to excretory columns.

FIGS. 12, 13. Levels of anal canal.

FIG. 14. Level posterior to anus.

PLATE IX

Ascaris lumbricoides larvae 24 hours in mouse liver (Figs. 1–5). H. and E. ×1800.

FIG. 1. Median sagittal section of the esophageal region from the nerve ring (left) to the esophageal bulb. Ganglionic nuclei fill the body cavity obscuring all other structures.

FIG. 2. Transverse section at a level of the esophageal isthmus. Ganglionic nuclei fill the body cavity.

FIG. 3. Transverse section of the esophageal bulb. The nucleus of the excretory cell is in a subventral position.

FIGS. 4, 5. Transverse sections at mid-intestinal levels. Excretory columns are absent and little can be seen of the intestinal cells. The lateral alae are minute.

A. lumbricoides larvae 36 hours in mouse liver (Figs. 6–8). H. and E. ×1800.

FIG. 6. Transverse section of esophageal isthmus near the level of the nerve ring. The esophagus has increased in diameter and can be differentiated from the ganglionic nuclei.

FIG. 7. Median sagittal section of intestine. Two intestinal cell nuclei are in the central body cavity.

FIG. 8. Transverse section at a mid-intestinal level. Minute lateral alae are difficult to see.

PLATE X

A. lumbricoides larvae 65 hours in mouse liver (Figs. 1–4). H. and E. ×1800.

FIG. 1. Longitudinal section at level of the esophageal bulb. The nucleus of the excretory cell is in the ventral body cavity. The nucleus of the dorsal esophageal gland defines the posterior esophageal bulb and is above and to the left of the excretory cell nucleus.

FIG. 2. Transverse section at level of the excretory cell nucleus. The esophagus is centrally located.

FIG. 3. Transverse section at a level of the esophageal isthmus. Ganglionic nuclei fill the body cavity.

FIG. 4. A longitudinal section showing one intestinal cell nucleus and a transverse section through an intestinal cell nucleus. Excretory columns are not present. Lateral alae are minute.

A. lumbricoides larvae 88 hours in mouse liver (Figs. 5–7). H. and E. ×1800.

FIG. 5. Transverse section at a level posterior to the nerve ring.

FIG. 6. Transverse section of the posterior esophageal bulb. The nucleus of the dorsal esophageal gland is in the dorsal sector.

FIG. 7. Transverse section at level of the anterior intestine. Lateral excretory columns are evident, the left column being the larger.

PLATE XI

A. lumbricoides larvae 88 hours in mouse liver (Figs. 1, 2). H. and E. ×1800.

FIG. 1. Longitudinal section of an intestinal level. The intestine has a prominent lumen and 2 cells are shown in the section. The excretory cell and dorsal esophageal gland nuclei are at the level of the posterior esophageal bulb (top).

FIG. 2. Longitudinal section of the tail showing the anus and anal canal.

A. lumbricoides larvae 5 days in mouse lung (Figs. 3–5). H. and E. ×1800.

FIG. 3. Transverse section at level of the nerve ring.

FIG. 4. Transverse section at level of the excretory cell nucleus.

FIG. 5. Transverse section at an anterior intestinal level. Small excretory columns are in the lateral lines. The lateral alae have a size similar to those of *T. canis* larvae.

PLATE XII

FIG. 1. Transverse section at mid-intestinal level of *A. lumbricoides* larva 5 days in mouse lung. Excretory columns are well defined structures in the lateral lines. Large lateral alae are characteristic of these third-stage forms. H. and E. ×1500.

FIG. 2. Transverse section at level of excretory cell nucleus of *A. lumbricoides* larva 6 days in mouse lung. H. and E. ×850.

FIG. 3. Transverse section at an intestinal level of *A. lumbricoides* larva 8 days in mouse lung. The excretory columns and central canaliculi are large and well defined. H. and E. ×650.

FIG. 4. Longitudinal section at an esophageal level of *A. lumbricoides* larva 8 days in mouse lung. The excretory cell covers the posterior esophagus. H. and E. ×600.

PLATE XIII

FIG. 1. Transverse section at level anterior to the nerve ring of *Necator americanus* 24 hours in mouse lung. Ganglionic nuclei surround the esophagus. Lateral alae are absent. H. and E. ×1800.

FIG. 2. Transverse section at level of nerve ring of *Necator* larva 2 days in mouse lung. Double lateral alae are present. H. and E. ×1800.

FIG. 3. Transverse section at level of one excretory cell nucleus of *Necator* larva 2 days in mouse lung. H. and E. ×1800.

FIG. 4. Transverse section at mid-intestinal level of *Necator* larva 2 days in mouse lung. Double lateral alae are prominent. H. and E. ×1800.

FIG. 5. Transverse section at level of glandular esophagus of *Necator* larva 3 days in mouse lung. H. and E. ×1800.

FIG. 6. Transverse section at level of posterior esophagus of *Necator* larva 4 days in mouse lung. An excretory column is in the lateral field. H. and E. ×1600.

FIG. 7. Longitudinal section at intestinal level of *Necator* larva 6 days in mouse lung. H. and E. ×600.

PLATE XIV

FIG. 1. Transverse section at intestinal level of *Necator* larva 6 days in mouse lung. The intestinal lumen is filled with particulate matter. Excretory columns are present in the lateral lines. H. and E. ×750.

Ancylostoma caninum larvae 24 hours in mouse lung. H. and E.

FIG. 2. Longitudinal section at level of the two excretory cell nuclei. ×900.

FIG. 3. Longitudinal section at level of the genital primordium. ×900.

FIG. 4. Longitudinal section of the anterior tip. ×1800.

PLATE XV

FIG. 1. *A. caninum* 7 days in mouse lung. Longitudinal section showing excretory cell nuclei and columns.

A. caninum larvae 30 days in mouse brain (Figs. 2–9). H. and E. ×1800.

FIG. 2. Transverse section anterior to nerve ring.

FIG. 3. Transverse section at level of glandular esophagus. Double lateral alae are prominent.

FIG. 4. Transverse section at anterior intestinal level. One large excretory column occupies the central body cavity.

FIGS. 5, 6. Transverse sections at levels of the excretory cell nuclei. The intestine has a small lumen and is centrally compressed.

FIG. 7. Transverse section at level posterior to the excretory columns.

FIG. 8. Transverse section at level of anal canal.

FIG. 9. Transverse section posterior to anus.

PLATE XVI

Strongyloides stercoralis larvae 7 days in mouse abdominal muscle (Figs. 1–3). H. and E. ×1470.

FIG. 1. Longitudinal section of esophagus posterior to the nerve ring. Esophageal glands surround the slender esophagus.

FIG. 2. Longitudinal section at level of the genital primordium.

FIG. 3. Longitudinal section at the esophago-intestinal junction.

FIG. 4. *Strongyloides* larva in lamina propria of human colon (Hartz' material). Transverse section just anterior to the nerve ring. The prominent triradiate esophagus with tubular expansions are characteristic of this level. H. and E. ×2150.

FIG. 5. Transverse section of larva in Fig. 4, at a level about equidistant between mouth and nerve ring. One nucleus is located in each field of the esophagus. H. and E. ×2150.

PLATE XVII

Strongyloides larvae in lamina propria of colon. (Hartz material.) H. and E. ×2150.

FIG. 1. Longitudinal section at level of posterior esophagus. The dense basophilic esophageal glands with 2 gland nuclei obscure much of the esophagus.

FIG. 2. Transverse section just posterior to the nerve ring. Ganglionic nuclei surround the esophagus.

FIG. 3. Transverse section at level of the two subventral esophageal gland nuclei.

FIG. 4. Transverse section at level of the posterior esophagus. The 3 esophageal glands surround the central esophagus.

FIG. 5. Transverse section at intestinal level. Two intestinal cells are closely opposed without forming a lumen.

FIG. 6. Transverse section at level posterior to the anus.

RESEARCH NOTE

TREATMENT OF A CASE OF *TAENIA SAGINATA* INFECTION WITH DIPHENHYDRAMINE

Diphenhydramine hydrochloride ("Benadryl hydrochloride") was demonstrated by Suing (1950, Brit. Med. J. **4657**: 822) to have some effect in eliminating oxyurid infections. On the assumption that this drug might also be effective against *Taenia* infections, it was tried on a case observed in the hospital of the Federal Correctional Institution, Seagoville, Texas. The drug, supplied through the courtesy of Parke, Davis & Company, Detroit, Michigan, was used in the form of an "emplet," a tablet coated to resist the acidity of the stomach for 2 hours and to free the active ingredient in about 30 minutes after reaching the alkaline condition of the small intestine.

A 23-year-old white male complained of vague abdominal discomfort, weight loss, and malaise. He stated that he had a tapeworm infection which had been unsuccessfully treated elsewhere. Physical examination revealed slight undernourishment but nothing remarkable. On December 17, 1950, he submitted a stool containing proglottids of *Taenia saginata*. On January 4, 1951, he was given a conventional treatment with Aspidium oleoresin. This proved unsuccessful and proglottids were again passed in a short time. On February 10, 1951, he was placed in a liquid diet and 2 days later he was given a magnesium sulfate purge. The following day he was given quinacrine (Atabrine) 0.4 grams at 8:00 A.M. and 12:00 noon and another purge at 2:00 P.M. A segment of the worm about 1 foot long was recovered but no scolex was found. By April 13, 1951, the patient again complained of passing many proglottids.

On May 10, 1951, and subsequently each morning for the next 7 days he received "Benadryl" emplets with consecutive oral doses in milligrams given as follows: 50, 150, 150, 200, 250, 300, 300 and 200. On the final day of drug treatment he also received Seidlitz powders as a purge given 2 hours after the "Benadryl." He complained at this time of drowsiness which persisted about 1 hour. Three hours after the purge was given he passed a portion of the tapeworm approximately 3 feet long. This appeared to be almost the entire worm although the scolex was missing. The worm was very sluggish in motion, possibly an effect of the drug. No proglottids were reported to be passed subsequently by the patient nor were they found in stool examinations done 67, 81, and 84 days after treatment. The patient gained weight, became more active and did not complain further of abdominal discomfort. He was not seen further.

We believe this case represents successful treatment of a *T. saginata* infection with "Benadryl" but we rarely encounter this tapeworm and have been unable to treat further cases. Our findings are presented for the benefit of those who may wish to try this drug against tapeworms.—ROBERT C. RENDTORFF, U. S. *Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Laboratory of Tropical Diseases, Columbia, S. C.* Present address: *University of Tennessee College of Medicine, 62 South Dunlap Street, Memphis, Tenn.*

PLATE I

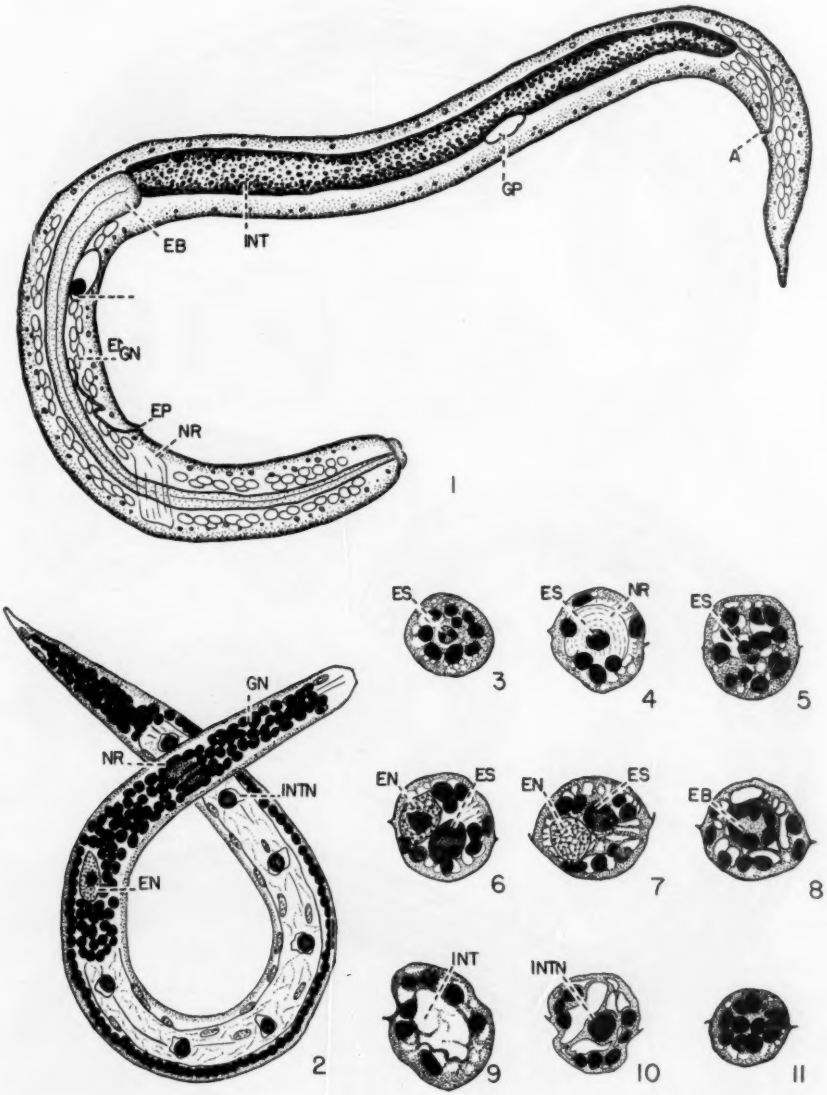
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PLATE II

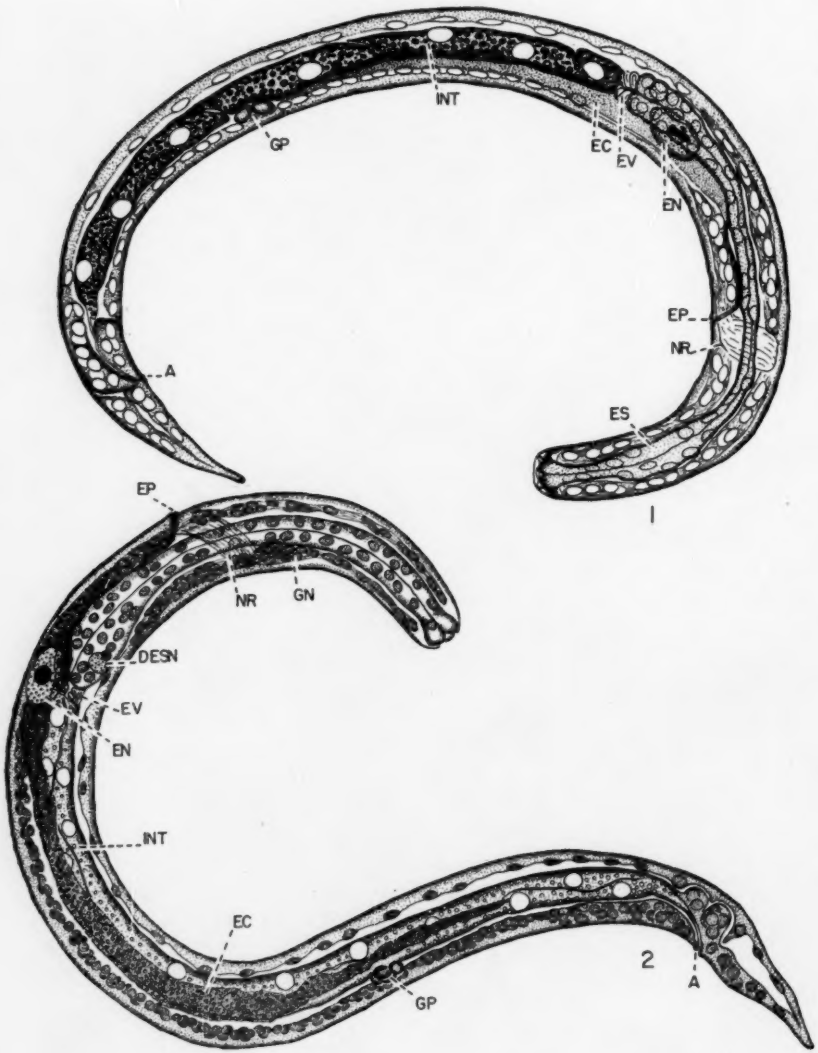

PLATE III

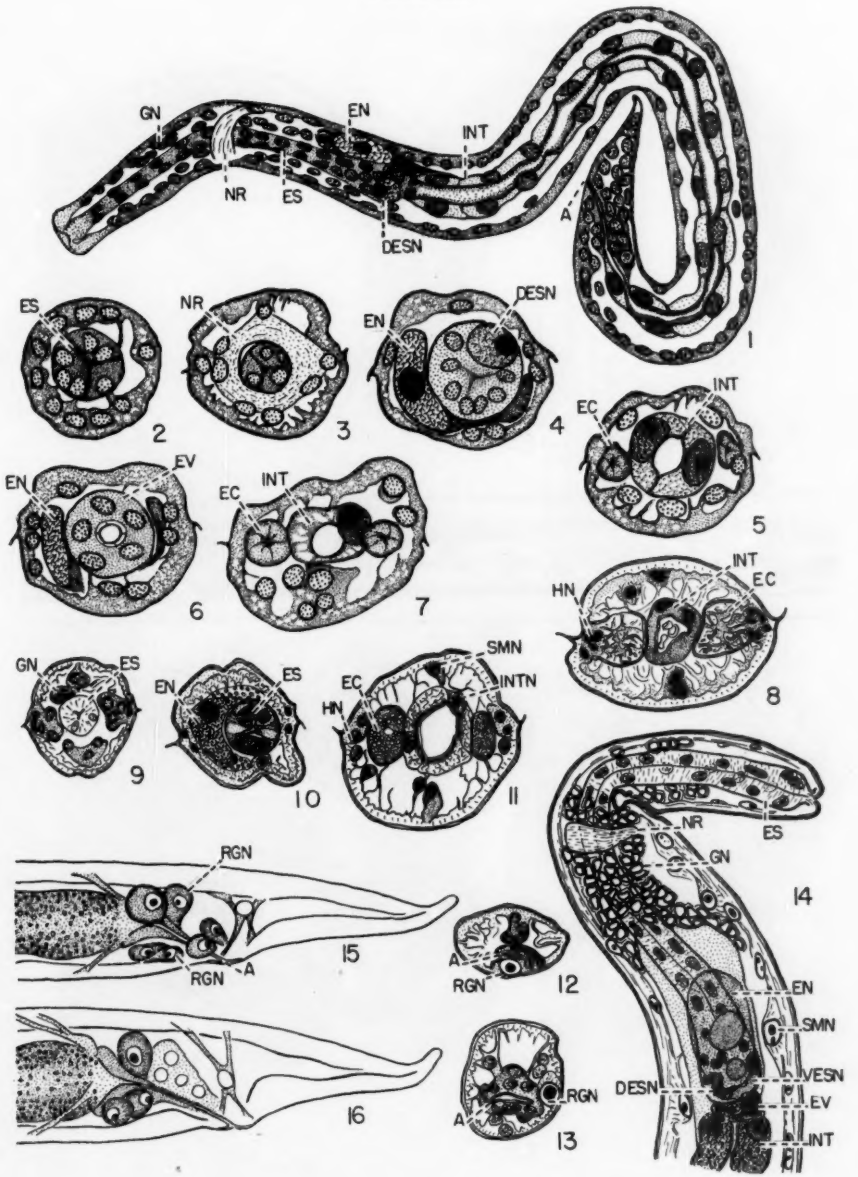
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PLATE IV

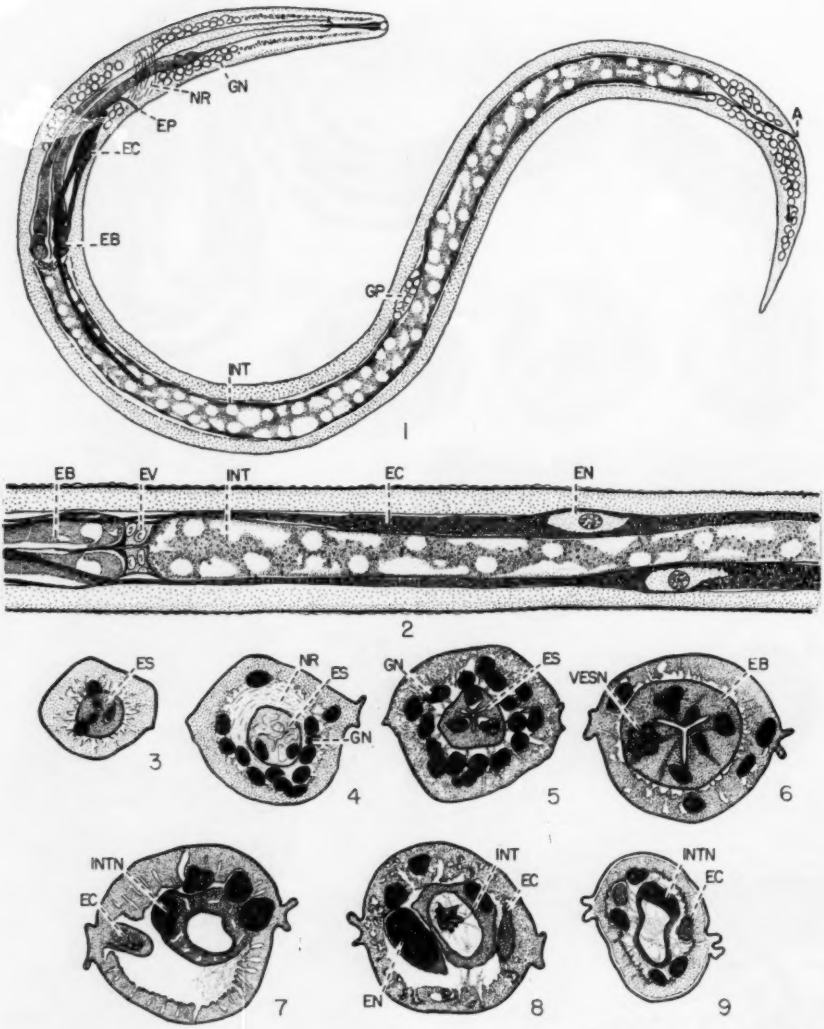
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PLATE V

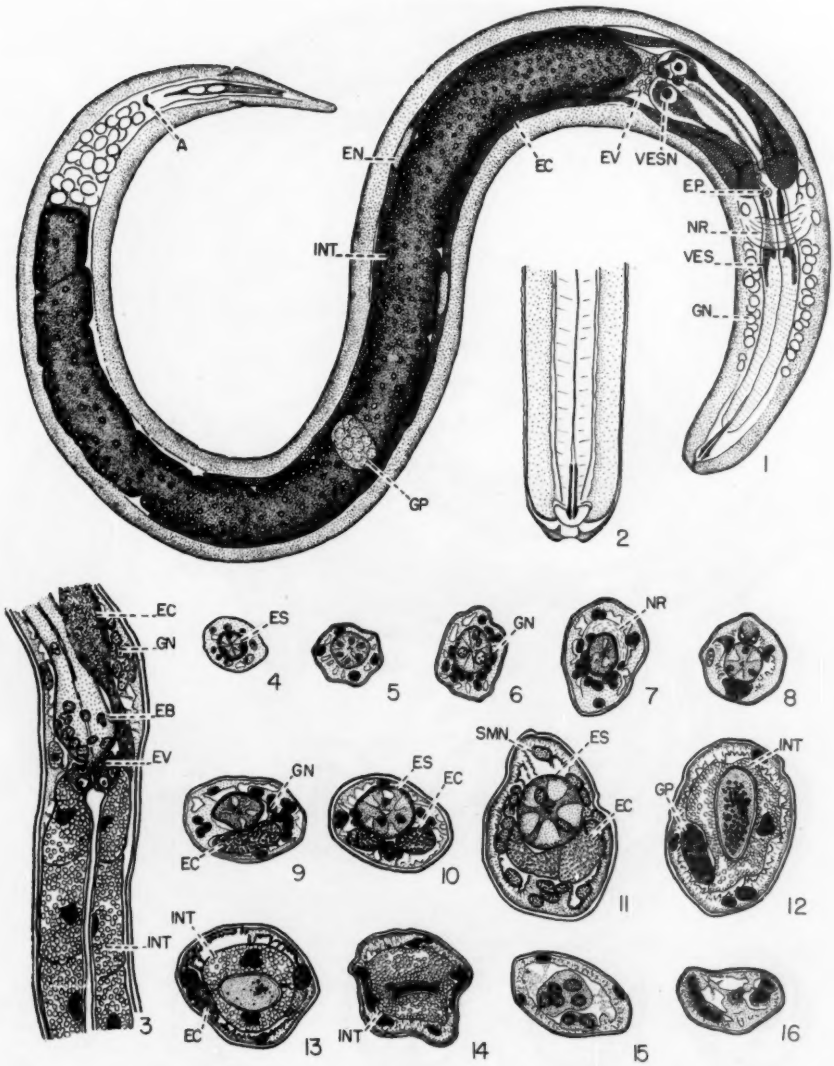
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PLATE VI

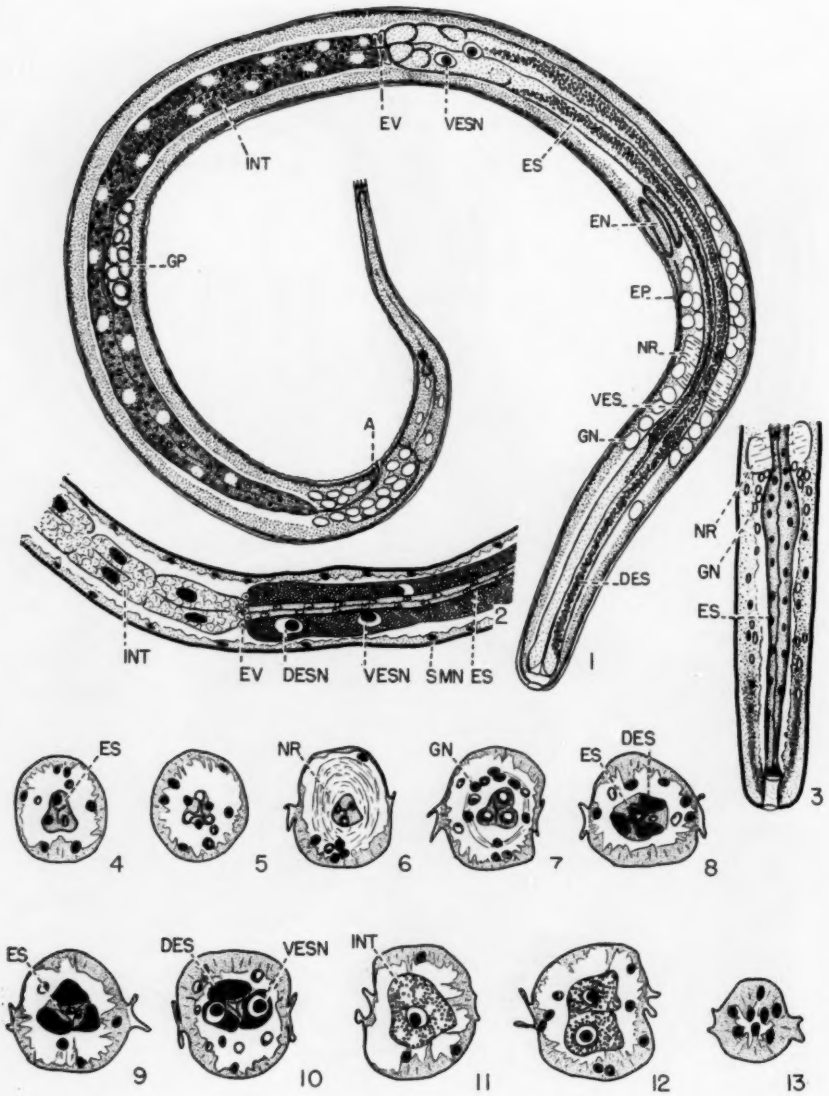
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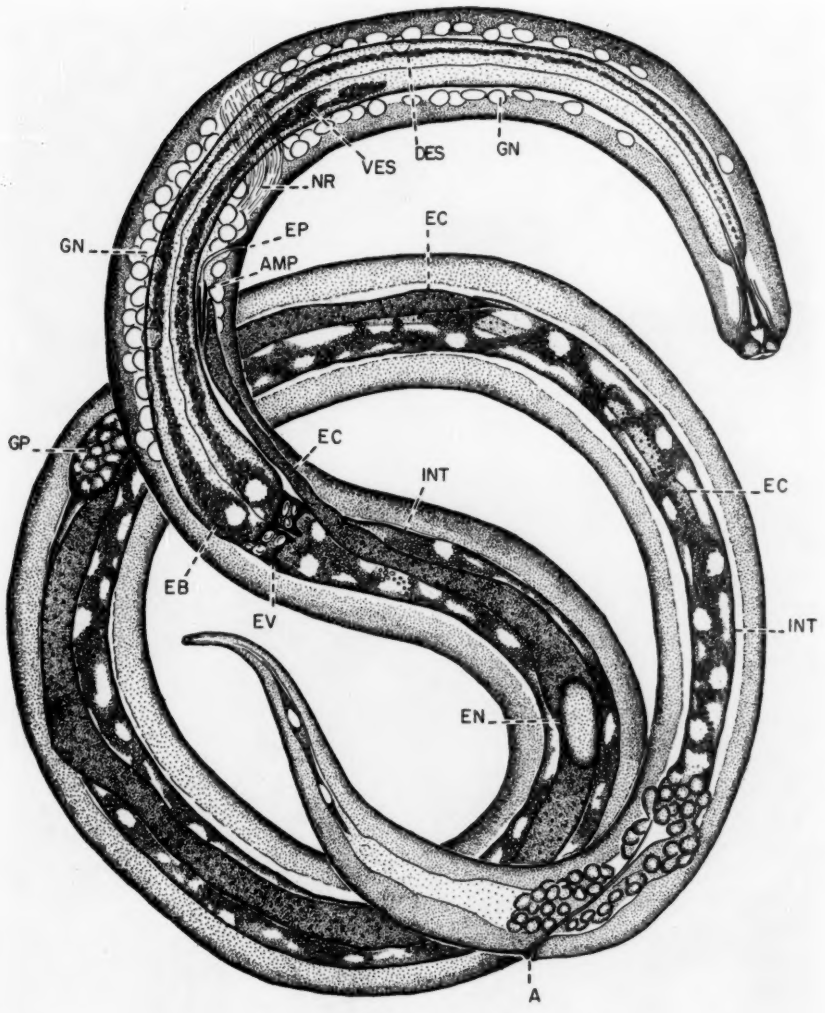
PLATE VII



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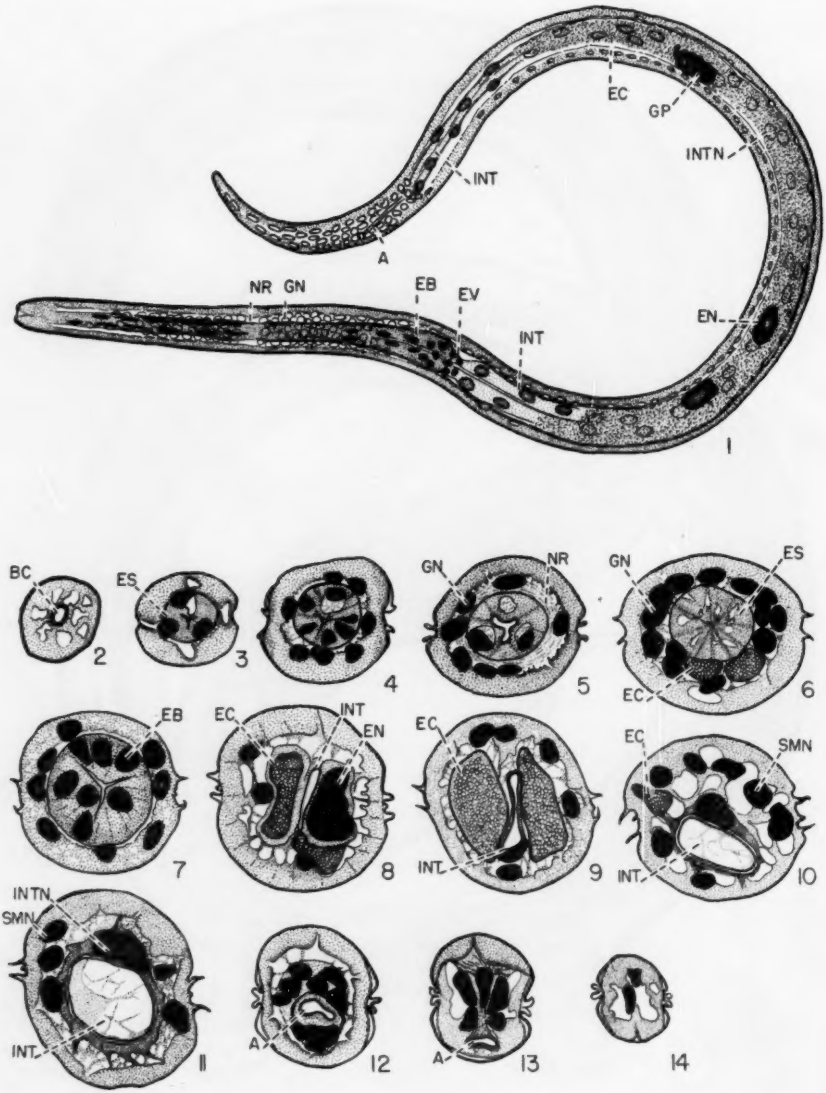
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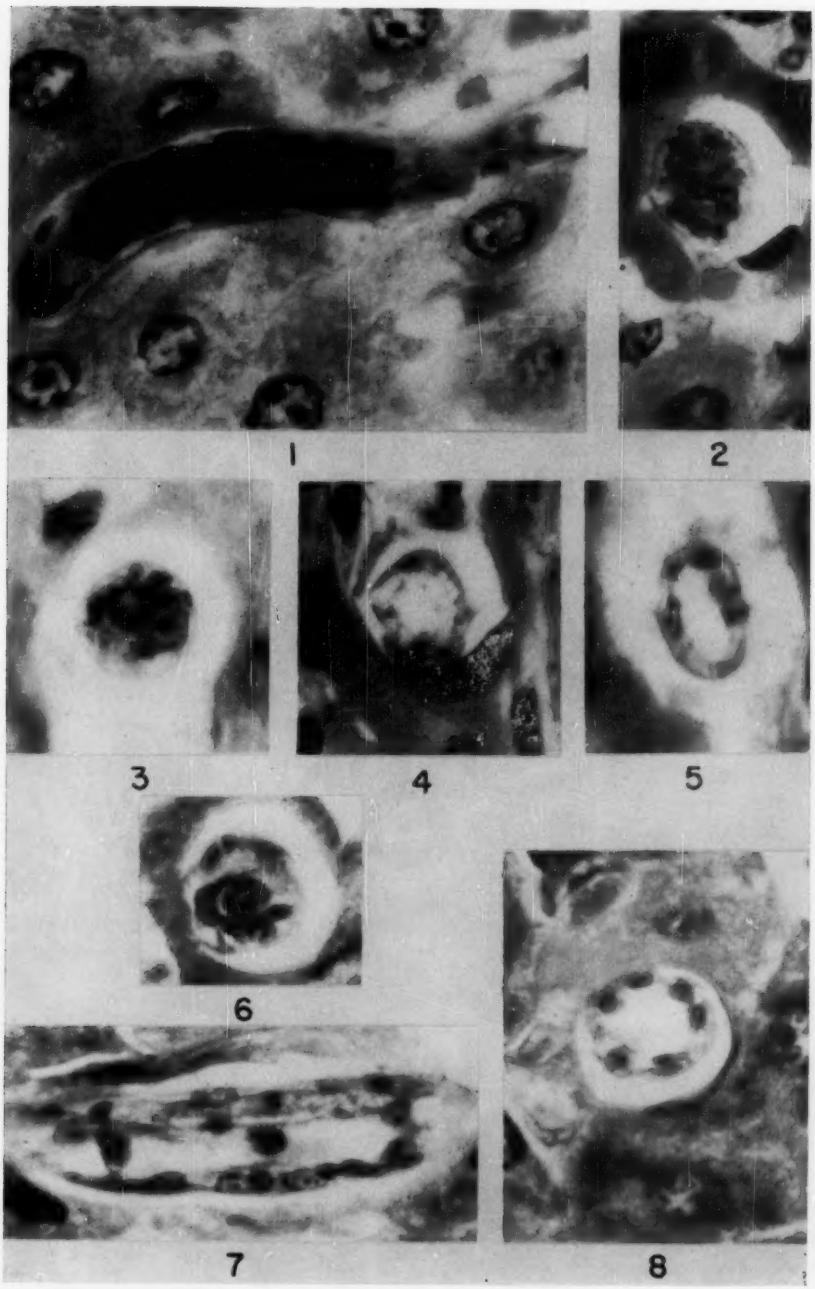

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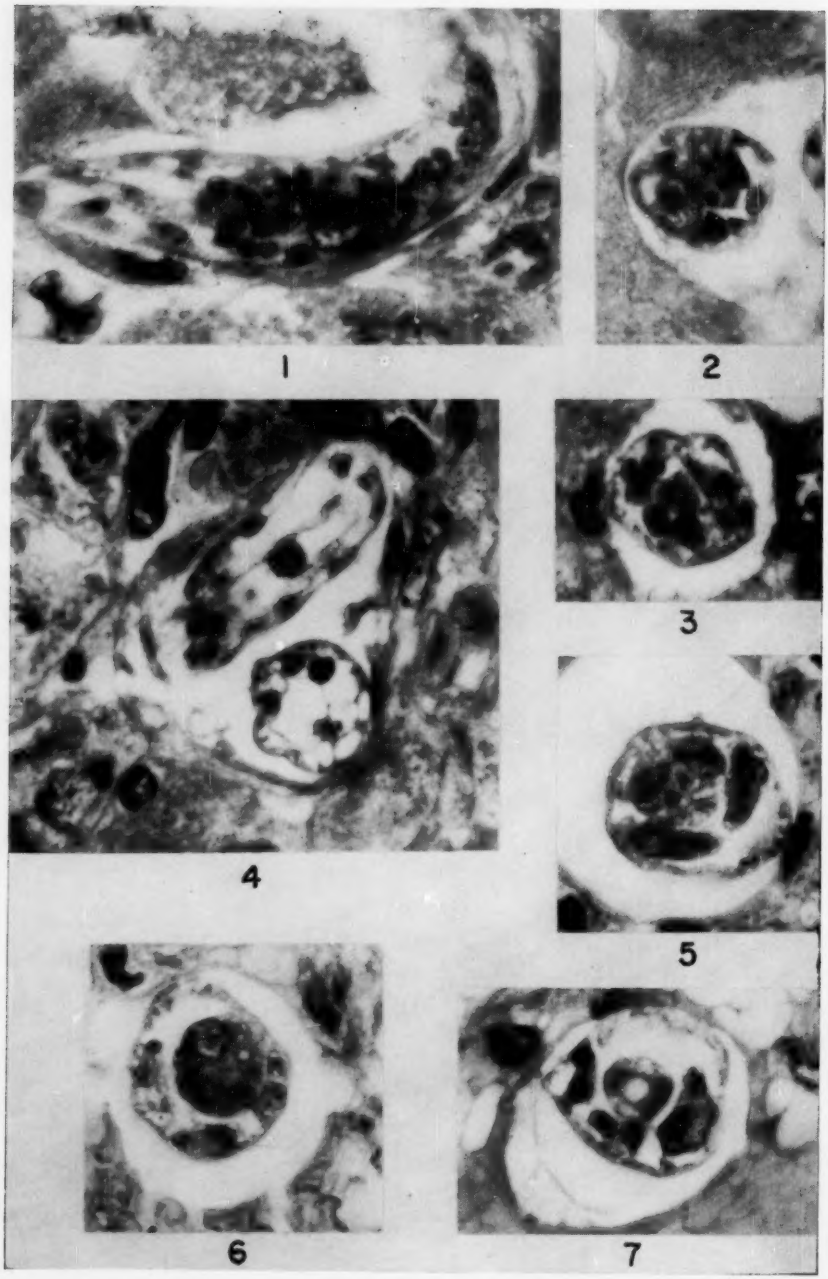

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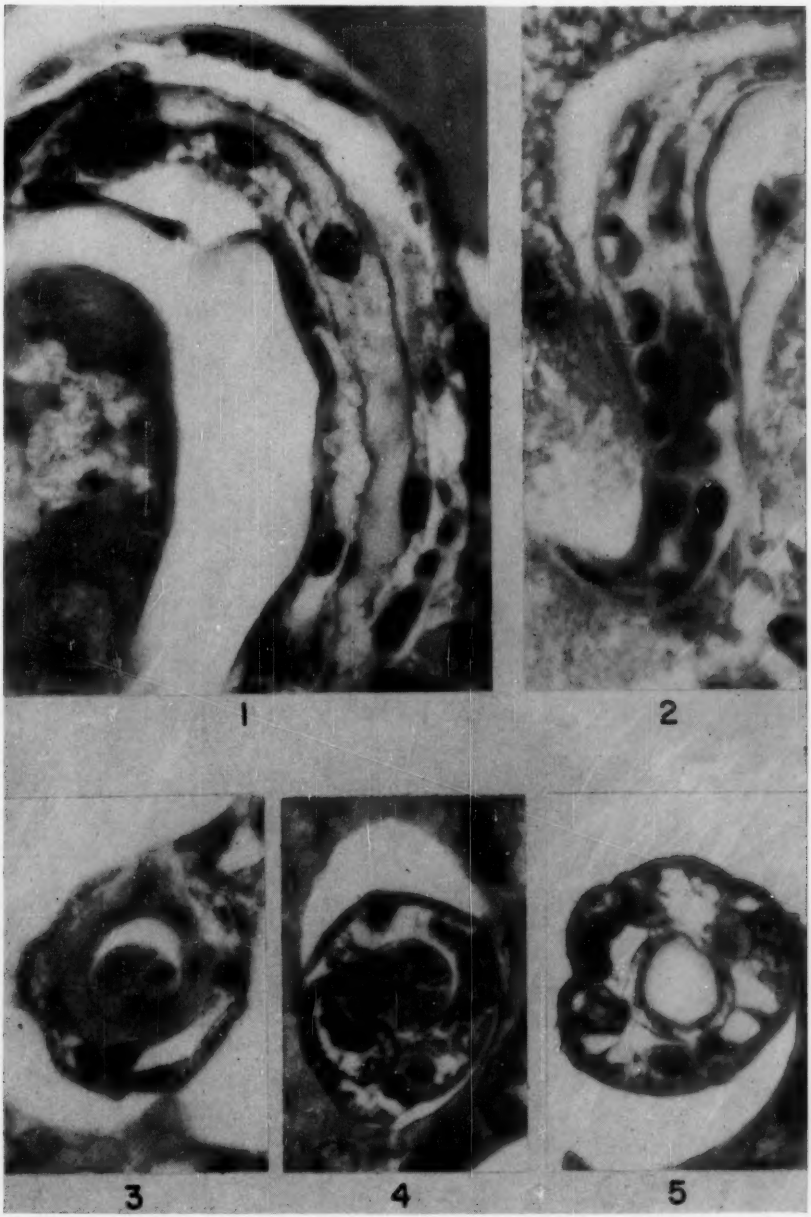

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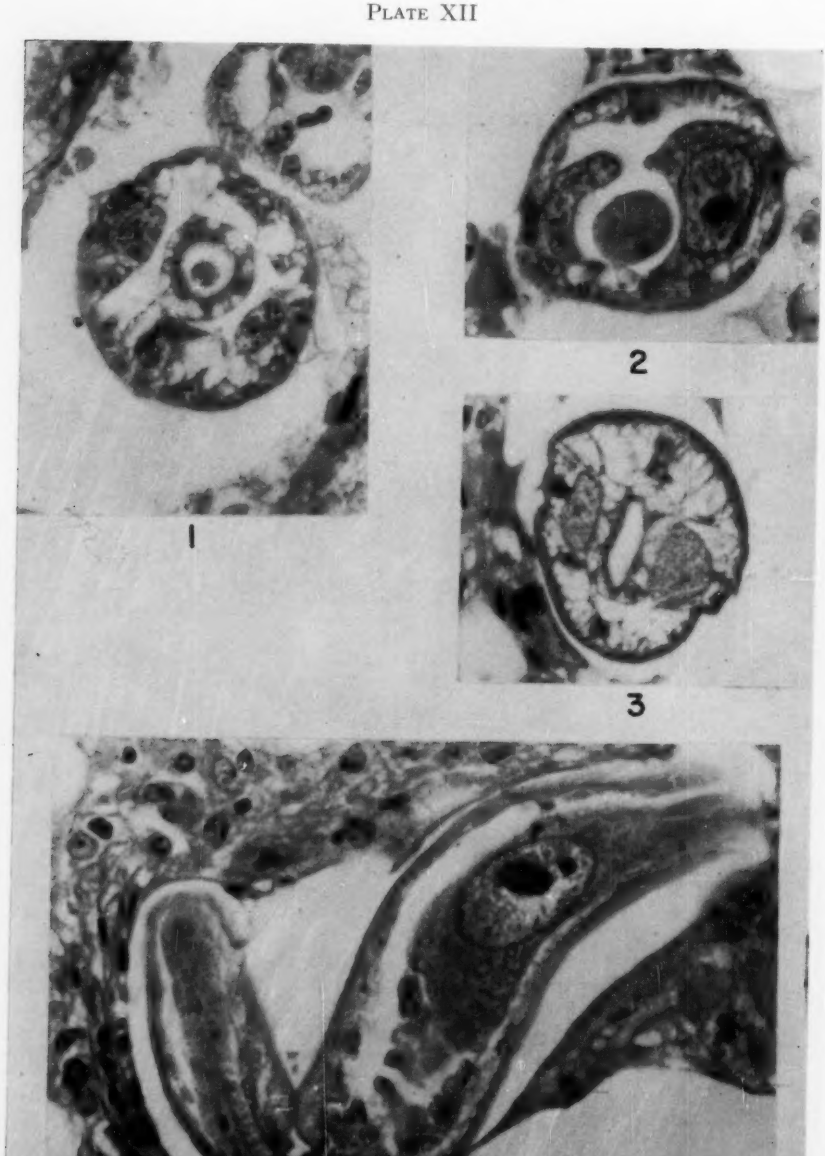

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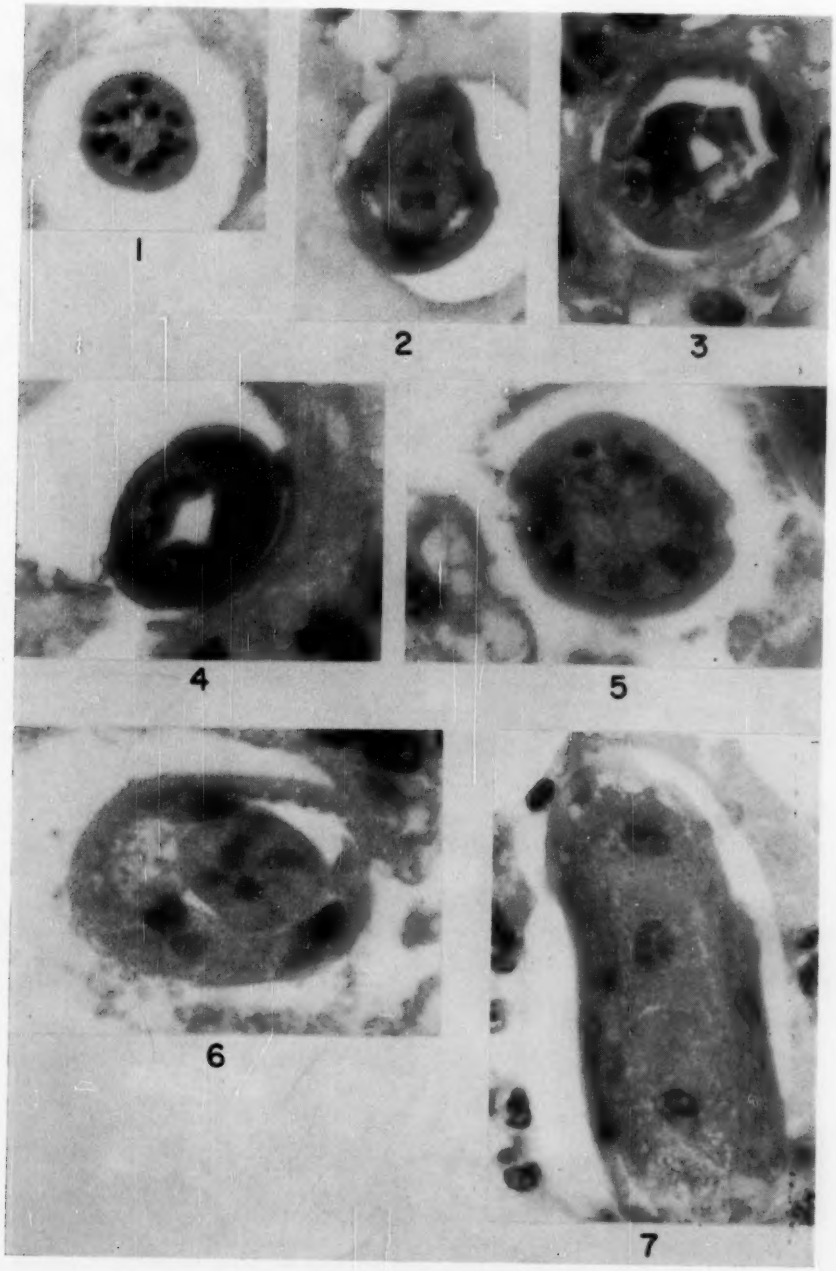

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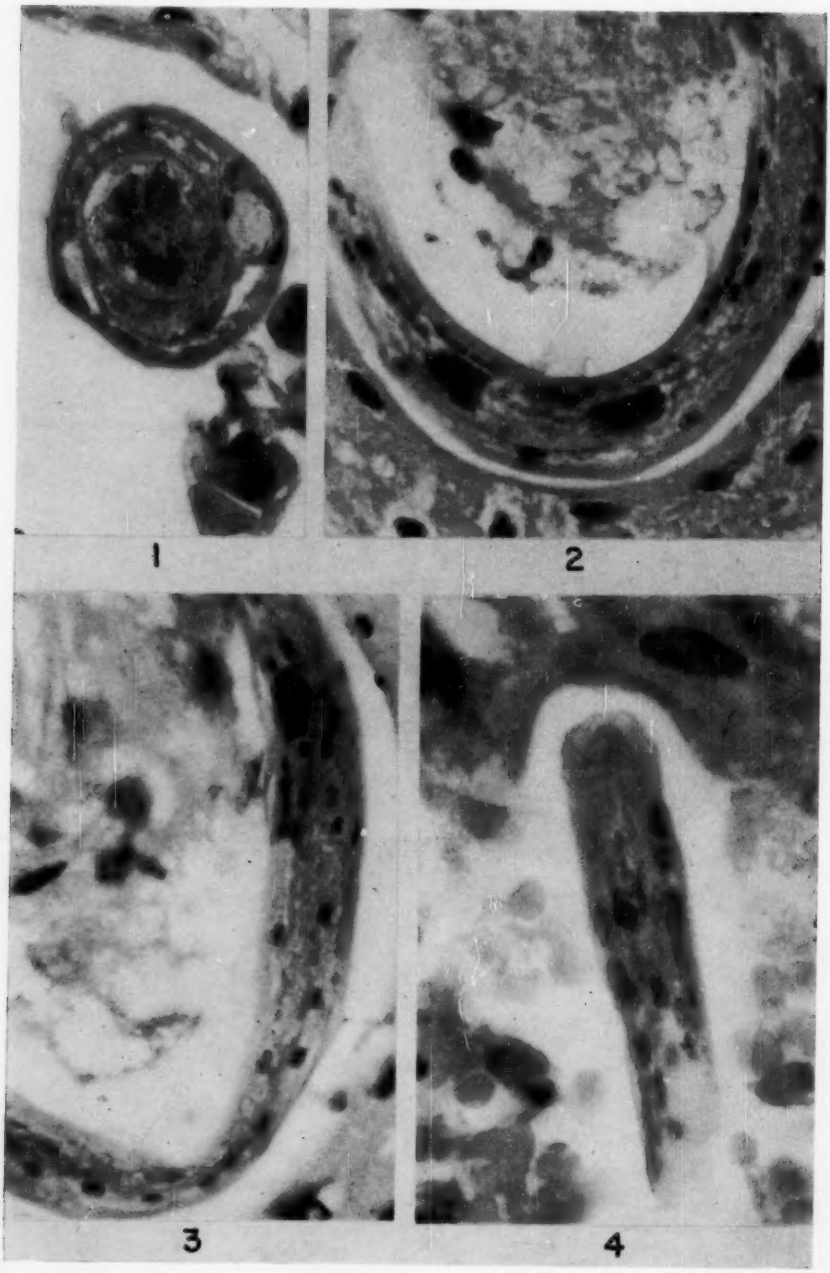

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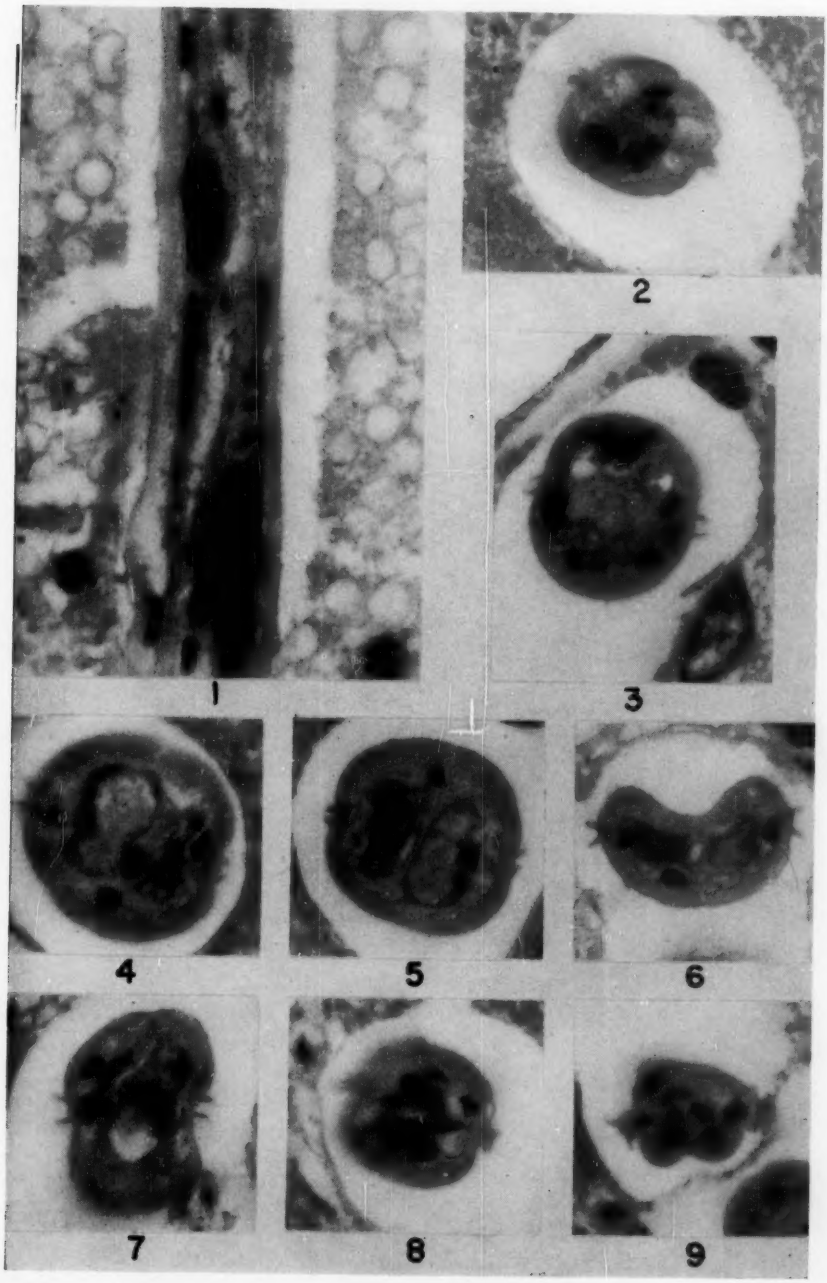

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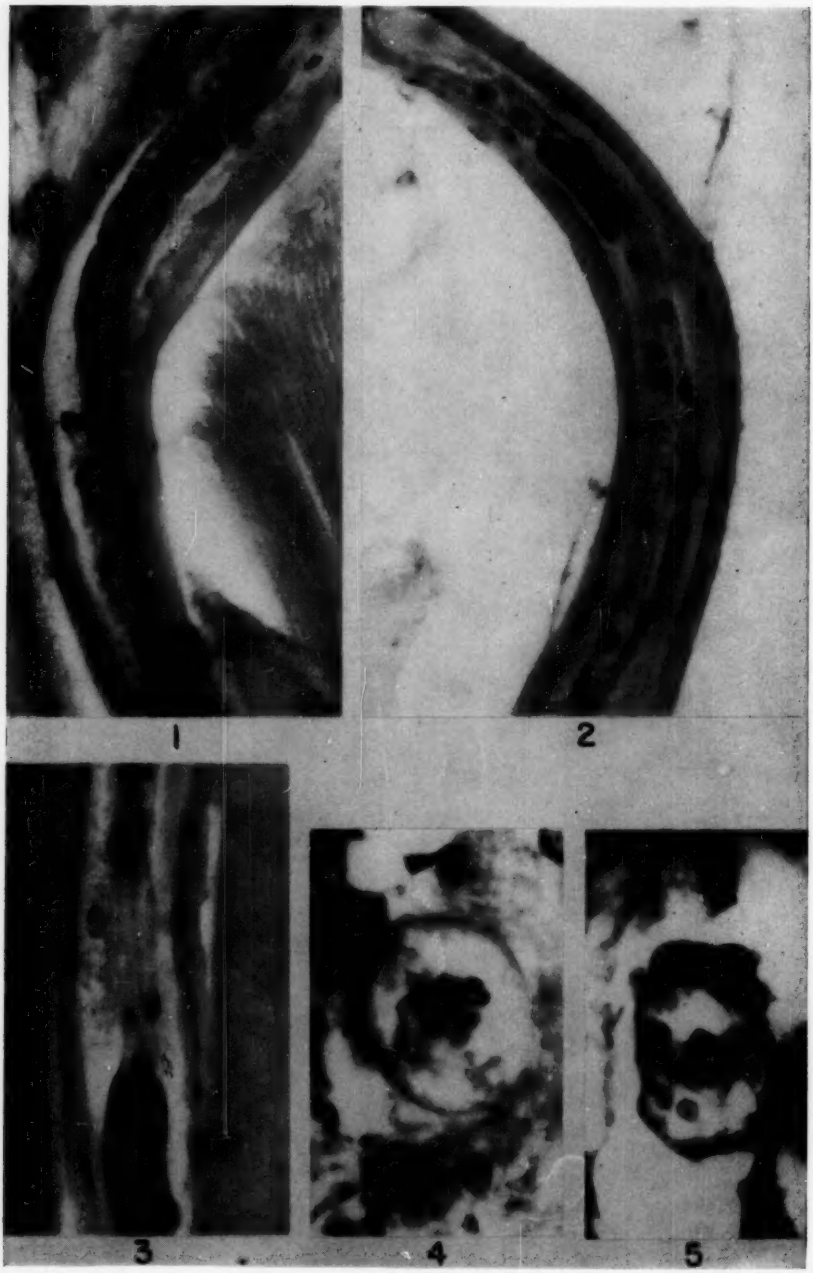

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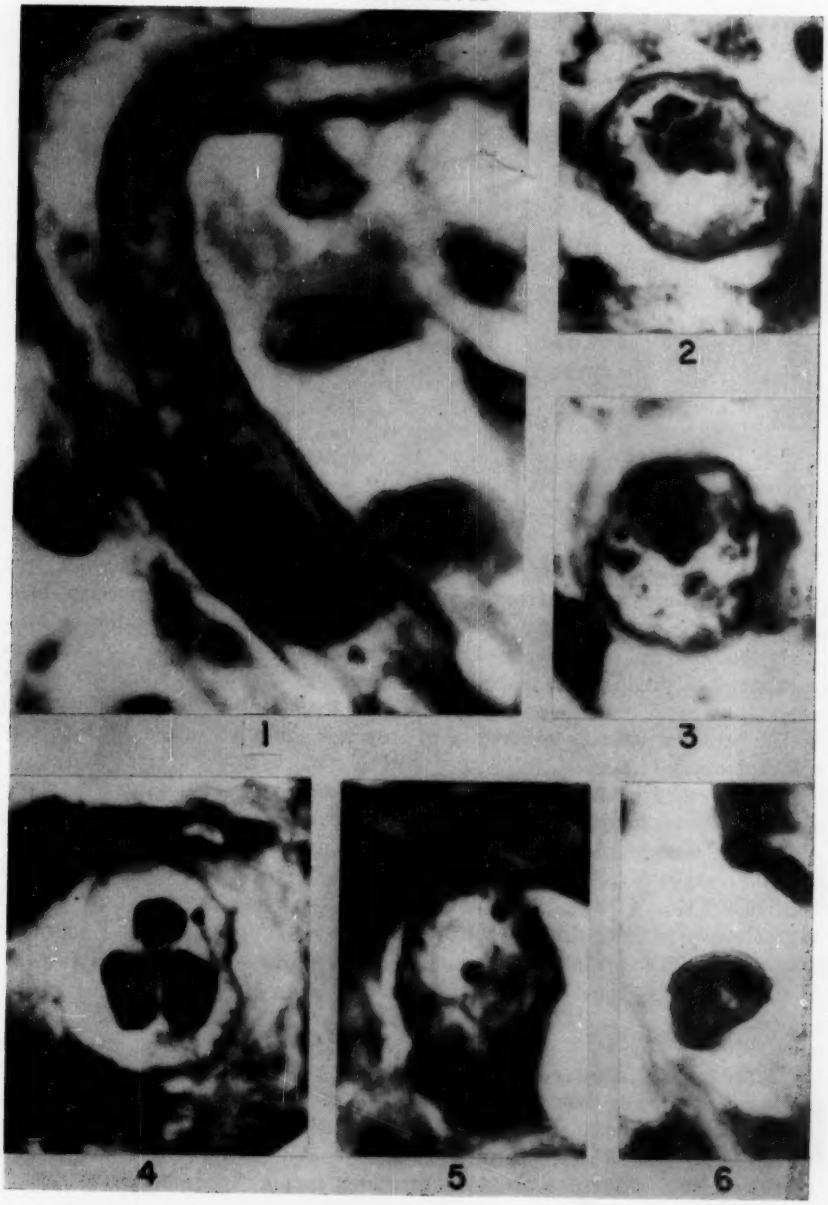

THE LIFE-HISTORY OF *PARAMACRODEROIDES ECHINUS* VENARD, 1941, A TREMATODE OF THE FLORIDA GAR, *LEPISOSTEUS PLATYRHINCUS*[1]

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University of Miami

A large species of xiphidiocercaria from the pulmonate gastropod *Helisoma duryi* was observed to penetrate and encyst in a variety of small fishes taken from South Florida canals. Its eventual transformation during encystment resulted in a metacercaria resembling a species of adult fluke seen earlier in the intestine of the Florida gar, *Lepisosteus platyrhincus*. Laboratory-reared metacercariae were fed to gar and the adults recovered were identified as *Paramacroderoides echinus* described from the intestine of the short-nosed gar, *Lepisosteus platostomous*, in Tennessee by Venard (1941). An abbreviated account of the life history of this species was published by Holliman and Leigh (1951). Venard placed this species in the family Macroderoididae (McMullen, 1937) but established *Paramacroderoides* as a new genus to contain it. It is still the only species in the genus.

MATERIALS AND METHODS

Helisoma duryi naturally infected and laboratory-reared and infected, was used as the source of cercariae for infection of the major fish intermediate host, *Gambusia affinis*. This fish was laboratory-reared or taken from artificial lakes known to be free of gar. Gar used as experimental final hosts were taken from an area in which the incidence of natural infection was low due to a scarcity of the snail host. In addition, they were kept unfed in the laboratory for a sufficient time to lose parasites naturally acquired or to provide a size differential for recognizing the experimental infection.

Measurements and drawings of adults and excysted metacercariae were made on stained and mounted specimens fixed in hot alcohol-formalin-acetic acid mixture. Cysts were measured in water without flattening after fixation with the above mixture. Cercariae were measured after fixation with hot 10% formalin after the method of Talbot (1936). Details of cercaria and sporocyst anatomy were worked out primarily on living material aided by the use of neutral red and Nile-blue sulphate. All measurements are given in millimeters.

LARVAL STAGES

Sporocysts (Fig. 6)

The eggs of *P. echinus* are fully embryonated and infective at the time of deposition as indicated by their ability to infect the snail host. Since embryonated eggs do not hatch spontaneously, ingestion by the snail was necessary in establishing all laboratory infections. Cercariae begin to emerge from laboratory-reared *Helisoma duryi* within 30 days after infection at summer temperatures.

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[1] This investigation was supported in part by research grant G 4051 C from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

Observations on the mother sporocysts and early developmental stages of daughter sporocysts were not made. However, the method of development appears to be similar to that described by Cort and Ameel (1944) for *Macroderoides typicus* (Winfield, 1929), a closely related form.

The ovoid daughter sporocysts showed definite signs of having developed in tubules formed from the mother sporocyst since they could often be dissected out in chains with the paletots of the sporocysts still connected by strands of tissue that might have been remnants of the original tubule as described for *M. typicus* (Fig. 6). This arrangement was most striking where the chains consisted of immature sporocysts, but the occasional sidewise attachments of sporocyst groups to a chain indicated the possibility of branching of the mother sporocyst tubule, an event not described for *M. typicus*. Immature sporocysts often occurred in the chains along with mature sporocysts.

Daughter sporocysts which are producing cercariae are a bright orange color due to heavy pigmentation of their paletots. This color often shows through the inner whorls of the host shell and makes it possible to determine infection by visual inspection. At 30–50 days of age, daughter sporocysts remain in masses of hundreds of sporocysts which mark the original location of mother sporocysts. These masses are usually adjacent to the host's esophagus, but have been seen slightly posterior to the stomach and may overlap the digestive gland.

Sporocysts vary greatly in size at this age and exhibit every stage of development from those containing only germ balls to those which contain one or two mature cercariae with one or two more approaching maturity. The largest 40-day-old sporocysts measured 0.528 by 0.185 and cercariae were seen to emerge through a birth pore located at one end. The sporocysts continue to grow during the course of the infection. Sporocysts from older natural infections range from 1.32 by 0.17 to 2.27 by 0.24.

Cercaria (Figs. 1, 7, 8)

The cercaria of *P. echinus* belongs to the Cercaria Ornatae group of the xiphidiocercariae, possessing a stylet in the oral sucker and a fin-fold on the tail. The body cuticle is covered with minute spines decreasing in size and number posteriorly. When freely moving, the body anterior to the ventral sucker is slender and tubular while posteriorly it is broad and flattened. Body measurements of 30 specimens fixed without pressure are: length 0.303–0.400 (av. 0.347); maximum width 0.083–0.117 (av. 0.103). The mouth is subterminal, located in an ovoid sucker 0.050–0.062 (av. 0.056) in width equipped with a stylet 0.032 in length (Fig. 8). A well-developed prepharynx precedes a muscular pharynx. No part of the esophagus or digestive tract could be seen. The ventral sucker is circular in outline and measures 0.038–0.043 (av. 0.040) in diameter. This species is unusual in that the penetration glands are unequal in number on the two sides. The glands, which occupy the middle one-third of the body, have a clear semi-transparent appearance which makes them contrast sharply with the granular opaque appearance of the rest of the body. One pair of small glands, which resist staining with neutral red and Nile-blue sulphate, is located on the anterior lateral border of the ventral sucker. Larger gland cells, 3 on the right side and 4 on the left, stain readily with the above intra-vitam stains and occupy lateral positions. Ducts proceed anteriorly in a single group on each side to the base of the stylet.

The excretory bladder is a slender sigmoid tube extending from the posterior level of the penetration glands to the caudal pockets at the base of the tail. Its lumen is barely visible under normal conditions and the wall consists of a layer of thick cells with coarse granular cytoplasm. Only the posterior one-fourth of the bladder distends and contracts as the contents discharge into the caudal pockets. The primary collecting ducts are dilated at their junction with the bladder and contain small dark granules. The main ducts pass laterally across the posterior penetration glands and divide into anterior and posterior branches. Flame cells were rarely seen. Failure to determine the excretory pattern is undoubtedly due to the general opacity of the parenchyma and to the presence of numerous orange-yellow droplets, 3–8 microns in size, dispersed throughout the body but especially abundant in the region posterior to the ventral sucker. In the developing cercaria these droplets appear first as fine granules, concentrated in two broad bands in the lateral regions of the body. The tail, equipped with fin-fold, measures 0.165–0.241 (av. 0.203) in length and is inserted subterminally on the ventral side adjacent to caudal pockets lined with a cuticula which is provided with minute spines.

Metacercaria (Figs. 2–4)

The metacercaria of *P. echinus* undergoes a rapid and striking transformation. Within 6 hours the narrow channel of the excretory bladder has expanded the body posterior to the ventral sucker into a balloon-like structure, pressing the surrounding parenchyma into a thin peripheral layer. The bladder may be extended as far as the anterior border of the ventral sucker. The layer of thick cells which covers the cercarial bladder has expanded into a thin granular layer. After 24 hours the stylet remains and outlines of penetration glands and ducts are evident. Many of the orange droplets, so numerous in cercariae, have disappeared. The swollen bladder has not yet taken on the opaque black appearance of the older metacercariae. All averages given below are based on the measurement of ten cysts. One-day-old cysts have a wall which consists of a single layer, measure 0.483–0.579 (av. 0.531) in length by 0.241–0.296 (av. 0.274) in width. These measurements are not significantly smaller than those of the mature metacercaria. Cyst formation around the swollen body provides an enclosure almost large enough for later maturation of the metacercarial stage.

After 6 days the bladder is still the most conspicuous feature of the anatomy (Fig. 2). The body is slender and tubular anterior to the ventral sucker, but still fragile and swollen posterior to the sucker. Two complete rows of small spines have started to develop in the oral sucker. The digestive tract is completely formed and the primary reproductive organs are recognizable. However, the two testes are at this time almost transversely located, the right one slightly anterior. The distention of the bladder has forced these organs to form in a thin peripheral layer of parenchyma. In life the bladder is packed with minute refractile, spherical granules which give it an opaque black appearance.

After 10 days most of the metacercariae have lost the balloon-like expansion of the posterior region and have slenderized to approximate the condition in the mature metacercaria. However, there is considerable variation at this period in the development of metacercariae from the same fish. A third row of minute spines has appeared on the dorsal side of the oral sucker anterior to the two complete rows appearing earlier.

Between 15 and 18 days, at summer temperatures, metacercariae achieve maximum development and become infective for gar. The major differences between 10- and 18-day-old forms are the increased differentiation of the reproductive organs and the considerable increase in the size of spines on the body and in the oral sucker (Fig. 4). Eighteen-day-old cysts have two walls, an inner thin tougher transparent one formed by the cercaria and an outer fragile granular layer of host origin that separates readily from the inner wall. Eighteen-day-old cysts measure 0.531–0.607 (av. 0.576) in length by 0.262–0.338 (av. 0.295) in width. Excysted fixed metacercariae measure 0.828–1.104 (av. 0.956) in length by 0.124–0.193 (av. 0.154) in width posterior to the ventral sucker. Measurements and observations on 74-day-old metacercariae indicate that no changes in size or morphology occur after 18 days and feeding experiments indicate that the 18-day-old forms are infective.

ADULT

Comparisons of the morphology of South Florida adults with Venard's (1941) description of *P. echinus* did not reveal any major differences (Fig. 5). There was some disparity in the size of eggs reported from Venard's preserved Tennessee specimens and the Florida material. Immature eggs from Venard's specimens were 0.039 by 0.025 with the older eggs measuring 0.036 in length. Living embryonated eggs from Florida *P. echinus* usually measured 0.044 by 0.023 and were clearly operculated.

Venard did not indicate the number of spines in the 3 rows attached to the oral sucker in his description of the adult. The two complete rows contain 43 or 44 to the row while the third anterior partial row on the dorsal side of the sucker contains 14 spines, making a total of approximately 101 spines.

The advanced development of the infective metacercariae enables some of the adults to achieve sexual maturity as early as 7 or 8 days at summer temperatures, but the eggs are few in number, incompletely embryonated and fail to infect snails. Worms 12 days old have reached maturity and contain numerous embryonated eggs which are infective for snails.

After 8 days the maturing adult worm averages about 1.19 in length by 0.143 in width at the ventral sucker; by 18 days it averages 1.64 in length by 0.258 in width. Adults taken from naturally infected gar have measured as much as 5.16 in length by 0.61 in width. A disproportionate amount of the increase in length in older specimens occurs posterior to the testes to accommodate the increasing bulk of the uterine coils.

All gar examined for natural infection were taken from a 20-mile portion of the Tamiami canal from 30 to 50 miles west of Miami, Florida. Most specimens were collected during the months from March to September in the years 1952, 1953 and 1955.

Incidence of infection for the 105 gar examined was 37%; 39 gar had infections ranging from 1 to 17 *P. echinus* per fish, with most infections consisting of fewer than 10 worms. Venard (1941) also found infections limited to small numbers; 8 of 10 gar examined carried infections varying from 1 to 15 individuals.

Another species of intestinal trematode of the same family found consistently during this study, often in large numbers, was identified as *Macroderoides spiniferus* Pearse (1924). Its life history, similar to *P. echinus*, has been worked out and will be published later.

DISCUSSION

Behavior of Cercariae

P. echinus cercariae emerge from the snail host after dark, most of them before midnight. They swim slowly with a wobbly side to side motion which, in general, moves them in an upward direction. There is no locomotor response to light. Brief periods of swimming alternate with rest periods during which they sink slowly with the body bent sharply at the ventral sucker into a U-shape with the ventrally inserted tail held between the two halves of the body (Fig. 7). The body posterior to the ventral sucker is greatly expanded, apparently serving as an aid to flotation. The dorsal curvature is held downward while settling and makes first contact with the bottom. This floating position may be maintained on the bottom for several seconds to several minutes before activity is resumed. Contact with the bottom does not result in any creeping motions.

Under the confinement of the isolation dish most cercariae, when undisturbed, remain suspended in a mass adjacent to the snail host by invisible strands of mucus. Since the mucus does not occur in the vicinity of uninfected snails, it is apparently of cercarial origin, and so long as cercariae are attached to the mass by threads of mucus they remain suspended with little activity almost indefinitely. Cercariae not so attached are engaged alternately in swimming and sinking motions. Kruidenier (1953) has studied the mucoid discharge from the caudal pockets of several species of non-virgulate xiphidiocercariae and suggested that the entanglements of cercariae in their accumulated mucus might ensure broader contacts with second intermediate hosts or aid in holding them in contact with a substrate in flowing water. The following observations support this suggestion.

Cercariae show no positive response to the presence of the vertebrate intermediate host until contact occurs. Attachment after contact is instantaneous and usually permanent. Penetration starts immediately and at the point of contact without exploratory crawling. Most cercariae penetrate the pectoral and caudal fins of the fish host, but this is due to the above-mentioned entangling of the cercariae in mucus to the active fins. The cercariae were observed to penetrate any region of the body except the eyes. In the latter location attachment occurs and penetration is attempted but not observed to be successful. Cercariae die in the act of penetration and the damage to the eye is repaired in 2 or 3 days.

Although penetration occurs most often in the fins, encystment does not occur there. As cercariae lose their tails and penetrate beneath the integument, there is an orientation towards the base of the fin and rapid internal migration in that direction. In approximately one-half hour the cercariae have migrated from all parts of the fin into the adjacent musculature. Since the fanning movements of the fins tend to collect most of the cercariae, encysted metacercariae become massed at the base of the fins where they are easily seen with the unaided eye.

As a typical example of localization in one *Gambusia* 35 mm. long exposed to 200 cercariae and dying after 5 hours, 56 cysts were removed from the base of the caudal fin, 18 from the heavily hemorrhaged muscle of one pectoral fin and 16 from the other. Forty-seven cysts were recovered from the rest of the body including 6 from the head, a total of 137 from the entire body, of which 66% were localized in the musculature of caudal and pectoral fins.

Apparently *P. echinus* is able to use a wide range of vertebrate intermediate

hosts. Small fishes in which encystment has been observed to occur normally are *Gambusia affinis, Jordanella floridae, Heterandria formosa, Mollienisia latipinna,* and *Fundulus* sp. Normal encystment also occurs in *Rana grylio* tadpoles and adult *Rana sphenocephala. Gambusia affinis* is the most abundant of the above fishes and is the most important of the intermediate hosts. Hunt (1952) found small fish to comprise 76.4% by volume of the stomach contents of 106 gar in S. Florida canals. *Gambusia affinis* was the fish most frequently encountered.

Pathology of Cercariae

The cercariae of *P. echinus* have killed *Gambusia* repeatedly when fish were exposed to sufficient numbers. The number that any fish can take and still survive is highly variable, depending apparently on the amount of internal hemorrhaging associated with penetration and encystment. Some *Gambusia* will succumb when exposed to 100 cercariae while others are able to survive exposure to 200 or more. Fish dying from exposure to cercariae usually exhibit obvious and sometimes massive areas of muscular hemorrhage. Death is occasionally preceded by loss of muscle control on one side while nearly normal control of the other side of the body is retained. Fins become badly frayed, and sloughing of the integument occurs over areas of penetration.

What at first appeared to be corneal damage to the eye sufficient to cause blindness proved to be temporary due to the failure of cercariae to complete penetration and the eventual reconstitution of the damaged tissues. The initial furrowing of the cornea is accompanied by a local reaction. Several cercariae can turn almost the entire cornea into a pustulous opaque layer which must result in partial or complete loss of vision temporarily. However, complete regeneration and clearing of the cornea occurs in several days.

It is doubtful that these cercariae are a significant mortality factor for small fishes in nature. Dissection of naturally infected fish reveal few metacercariae, rarely more than 10 per fish. From one collecting area 50 *Gambusia* were artificially digested and 182 *P. echinus* cysts recovered, an average of less than 4 per fish. Since laboratory exposure results in fish carrying over 50 metacercariae successfully, it is not likely that fatal infections occur often in nature.

Infecting the Gar

During early observations, unknown numbers of metacercariae were administered to gar by feeding infected *Gambusia.* Later, known numbers of metacercariae were placed by pipette directly in the stomach of the gar in order to ascertain rate of survival in the final host. Highly variable rates of worm survival from 0 to 52% were noted in the first 8 fish infected. Since these fish had been in the laboratory for several days to several weeks without regular feeding and were not fed regularly after infection, it is possible that successful excystation and attachment may be dependent on the amount of food present and the intestinal emptying time of the fishes. Also, survival may be affected by frequency of feeding.

The final gar infected in this study provided some indication that feeding before and after infection is related to successful infection. Three gar, 29–31 cm. long, had been unfed for 48 days. Two received 100 metacercariae (52 days old) each and were sacrificed after 72 hours. Two *P. echinus* were recovered from one host and none from the other. The third gar was fed on uninfected *Gambusia* for sev-

eral days prior to infection up to one hour before 35 metacercariae (37 days old) were placed in its stomach. Twenty-six of the 35 (74%) were recovered 7 days later, during which time daily feeding had taken place.

The following observations add further weight to the above suggestion. First, it was noted that the majority of *P. echinus* are in the posterior one-third of the intestine, although an occasional one is found in the anterior portions. Finally, there is the suggestion of rapid digestion and elimination in the observation of Hunt (1952) that only 24% of the gar examined in the field had food in their stomachs regardless of the time of collection. It may be that metacercariae do not have time to excyst and attach unless introduced into a full stomach.

SUMMARY

Helisoma duryi, a common pulmonate snail in South Florida canals, is frequently infected with a large xiphidiocercaria which penetrates and encysts in small fishes, usually *Gambusia affinis.* In 15–18 days encysted metacercariae are infective for the Florida gar, *Lepisosteus platyrhincus,* and develop within 9–12 days into sexually mature adults identified as *Paramacroderoides echinus* Venard, 1941.

Eggs of the trematode are embryonated and infective when deposited but hatch only after ingestion by the snail host. Daughter sporocysts in approximately 30 days start producing cercariae that are unusual in that there is an unequal number of penetration glands on the two sides of the body. Tadpoles, frogs and several genera of small fishes are suitable intermediate hosts.

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EXPLANATION OF PLATE

(Figs. 1–5 and 8 drawn with the aid of a camera lucida).

FIG. 1. Cercaria, ventral view.

FIG. 2. Metacercaria, 6 days old, showing greatly swollen excretory bladder.

FIG. 3. Metacercaria, 18 days old, in cyst; the granule-filled excretory bladder is still a conspicuous feature.

FIG. 4. Metacercaria, 18 days old, removed from cyst showing slenderizing of the body and degree of maturation of reproductive organs.

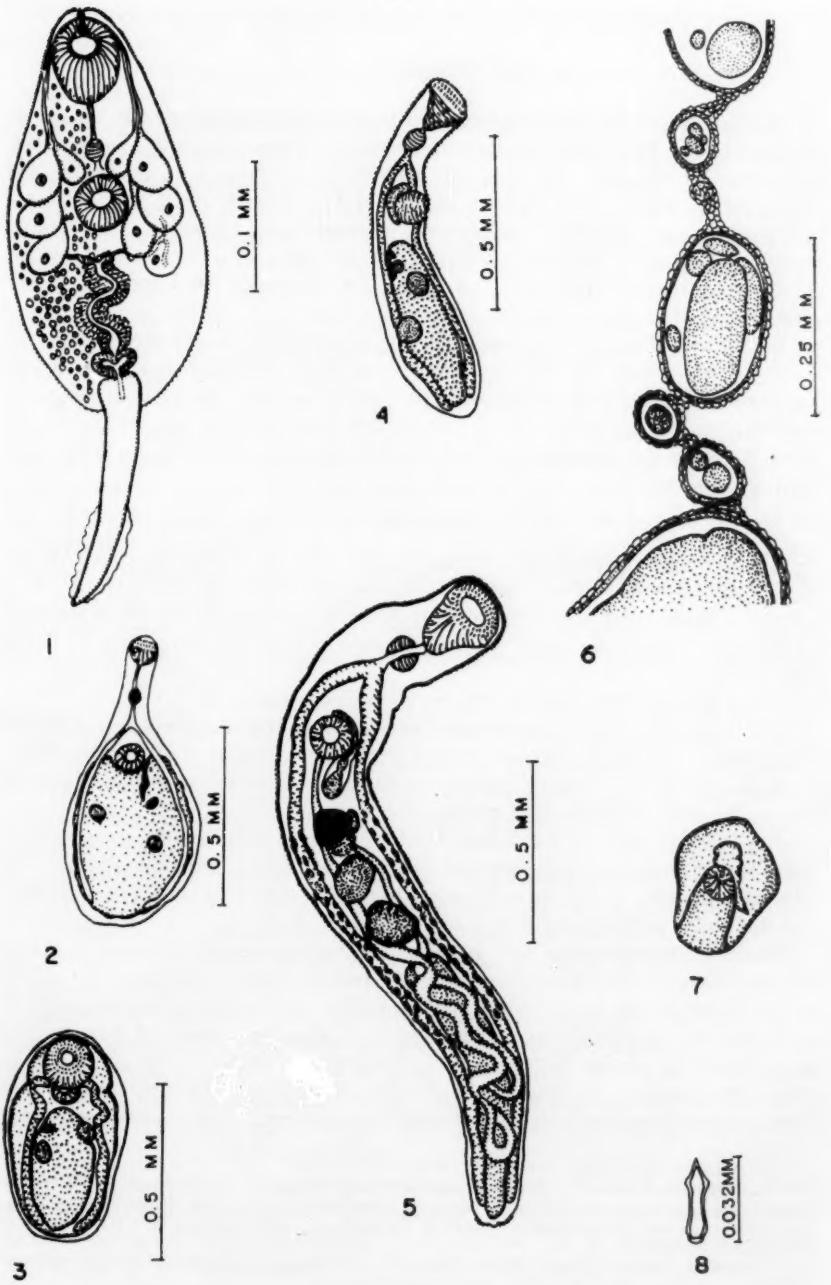
FIG. 5. Adult, 18 days old, uterus somewhat generalized.

FIG. 6. Daughter sporocysts, 40 days old, showing various stages of development in a single chain formed by attachments through extensions of their paletots. A tracing of enlarged photomicrograph.

FIG. 7. Cercaria, free-hand drawing, showing resting position.

FIG. 8. Stylet of cercaria.

PLATE I



PRESERVATION OF *TOXOPLASMA GONDII* BY FREEZING*

DON E. EYLES, NELL COLEMAN, AND D. J. CAVANAUGH†

Most investigators employing the conventional rapid freezing methods in alcohol-dry ice mixtures have failed in efforts to preserve *Toxoplasma gondii* (Sabin, 1938, and a number of others including this laboratory). Manwell *et al.* (1945) did report partial success, but preservation was not demonstrated for more than a few minutes nor were the results consistent. Weinman and Chandler (1954) mentioned preserving *T. gondii* for 60 days at a temperature of −70° C., but provided no details of the technique used. Recently, Dr. Weinman has informed us that glycerol was used and that he has a paper on this subject in press.

In our laboratory we have recently been successful also in the slow freezing of *T. gondii* in glycerol solutions. Our success has been consistent in contrast to our unsucccessful results with rapid freezing. To date we have shown that organisms may be preserved for periods up to at least 209 days, and it is the purpose of this paper to outline the methods employed and describe some of the factors influencing the degree of success.

METHODS

The techniques followed were basically those of Polge and his associates (Polge *et al.*, 1949 and many subsequent papers) who discovered the protective effect of glycerol. More specifically, we used some of the methods of Levine and Marquardt (1954) who successfully preserved the protozoan, *Tritrichomonas foetus,* by freezing.

The following steps were carried out in our experiments:

1. Peritoneal exudate containing *Toxoplasma* in large numbers was collected from infected laboratory mice and diluted 1:5 with a solution of 20 percent serum in physiologic saline. One ml. samples of the diluted exudate were then placed in thin-walled glass ampoules of 2 ml. capacity or more.

2. To each ampoule was added 1 ml. of glycerol solution, the concentration being varied according to the purpose of the experiment. After the glycerol was added the contents of the ampoules were thoroughly mixed by means of a syringe, and the necks carefully sealed in a bunsen burner flame.

3. The ampoules were then frozen. The various procedures used to obtain different rates of freezing are described in the results section of this paper.

4. After storage in the frozen condition either in the mechanical freezer or in an alcohol-dry ice mixture for varying lengths of time, the contents of the ampoules were thawed by placing the ampoules in a water bath at 37° C. for about 5 minutes. The contents, or a part of the contents, were injected intraperitoneally into clean mice to determine if viable organisms were present. The efficiency of preser-

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vation was estimated on the basis of the survival time of mice inoculated and, in some instances, by measuring the reduction in the titer which produced 50 percent mortality in inoculated mice.

FINDINGS

Initial experiments.

Two experiments demonstrated that *Toxoplasma gondii* could be frozen successfully and these served as a base line for further experiments in which the influence of various factors upon the efficiency of preservation was studied.

In these experiments, exudate was prepared as described in the methods section and was mixed with an equal quantity of 2M glycerol in distilled water (18.4 percent by weight). After sealing, the ampoules were placed in a test tube basket which had been lined with a layer of absorbent cotton approximately one inch in thickness. This basket was closed with a cotton plug and placed in the mechanical freezer at about – 20° C. Samples were thawed after one day and subsequently to determine the infectivity to mice.

In all instances the organisms were successfully frozen, but there was a great initial loss of infectivity. Prior to freezing the exudate in glycerol produced infection when diluted 10^6 or more times, but after freezing the infective titer of the exudate was at the most 10^3. This would indicate that only a very small proportion of the organisms withstood the freezing procedure; however, in spite of the loss of infectivity, samples from these first experiments produced infection after 128 days' preservation (the maximum tried), the ampoules being kept all this time at – 20° C. in the mechanical freezer.

Other experiments were then conducted to study the influence of the rate of freezing, the temperature of storage, and the proportion of glycerol. The results of these follow.

Influence of the rate of freezing upon the preservation of Toxoplasma.

One series of experiments was conducted in which varying rates of freezing were employed, other factors being maintained the same. Exudate was prepared as described in the methods section and 2-molar glycerol solution in distilled water was used.

Aliquot samples were frozen by each of the following methods. Curves illustrating the various rates of freezing thus produced are shown in Figure 1.

Method A. Ampoules were immersed directly into the dry ice-alcohol freezing mixture. Freezing temperatures were reached within 30 seconds and – 60° C. within 2½ minutes.

Method B. Ampoules were wrapped in cotton then placed in the center of 25 mm. test tubes. These were immersed in the dry ice-alcohol mixture. The interior of the ampoules reached – 16° C. in about 15 minutes. When this temperature was reached, the insulating tube was removed and the ampoules placed directly in the freezing mixture so that a temperature below – 60° C. was quickly reached.

Method C. Ampoules were placed in a naked test tube basket in a compartment in which dry ice is kept in the mechanical freezer. The temperatures slowly fell to – 18° C. over a period of 36 minutes. At this time, the ampoules were placed in the freezing mixture which brought them to below – 60° C. in a very short time.

Method D. Ampoules were placed in a test tube basket which had been completely lined with a layer of absorbent cotton approximately one inch in thickness.

This basket was placed in the dry ice compartment of the mechanical freezer. Temperatures fell very slowly, – 14° C. being reached only after 70 minutes and – 41° only after 160 minutes. After 48 hours, the ampoules were removed from the basket and placed directly in the dry ice-alcohol freezing mixture.

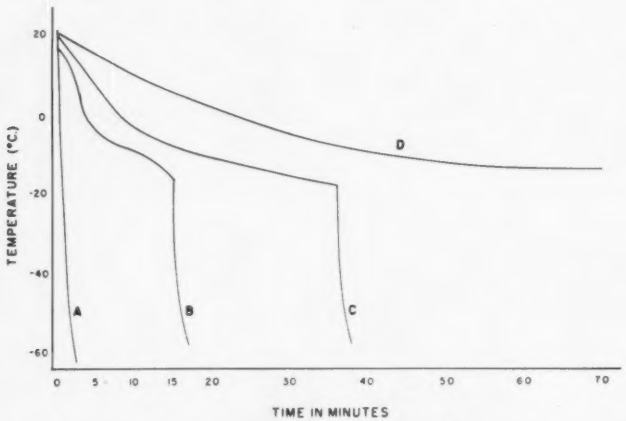

FIG. 1. Rates of freezing of contents of ampoules handled by different methods as described in text.

Specimens frozen by the four methods outlined were thawed after 48 hours and 0.1 ml. of the contents injected into each of five clean mice. The efficiency of preservation was estimated on the basis of the mean time to death of the lots, as it has been shown that the survival time of mice is inversely related to the number of organisms injected (Eyles and Coleman, 1956). The results were as follows:

Method A. No mice died
Method B. 8.4 ± 0.0 days
Method C. 7.6 ± 0.55 days
Method D. 7.2 ± 0.45 days

From the above it is evident that rapid freezing by direct immersion in the dry ice-alcohol mixture resulted in complete loss of viability. At the other extreme the method which gave the slowest rate of freezing produced inocula causing death in the least time. The intermediate rates of freezing gave intermediate results. From these experiments it would appear that the slowest rate of freezing produced the best results.

Influence of the temperature of storage.

Frozen samples have been kept either in the dry ice-alcohol freezing mixture at below – 70° C. or in the mechanical freezer at about – 15 to – 20° C.

Results of storage on infectivity may be assessed by consideration of the changes of mean time to death of lots of mice inoculated with frozen material. Table 1 shows these data for several frozen batches stored under various conditions. The first group of the table was stored in a mechanical freezer the temperature of which was between – 15 and – 20° C. but which fluctuated in temperature due to

the fact that it was frequently opened to procure materials. The survival time of inoculated mice became progressively greater and after the fourth day the batch was no longer infective.

The second group was stored in another mechanical freezer which was seldom entered and thus maintained a consistent temperature of about −20° C. In this case infectivity was maintained for 128 days (the maximum tried) but survival time tended to become greater as storage continued thus indicating deterioration of the material.

The last two groups were stored in the dry ice-alcohol freezing mixture in a Dewar flask. Little change in infectivity was indicated by the observations spanning the eighth through the 209th day.

These observations point to the conclusion that storage at the very low temperatures of the dry ice-alcohol mixture is best for preservation with the least loss of viability and subsequent observations have supported this hypothesis.

TABLE 1.—*Influence of the method of storage upon the viability of frozen* Toxoplasma gondii *as indicated by the time to death of mice inoculated with stored material*

Method and temperature of storage	Survival time in days of mice inoculated with material stored for varying lengths of time								
	1 day	2 days	4 days	8 days	16 days	32 days	64 days	128 days	209 days
In mechanical freezer at −15 to −20° C. but fluctuating	6.8	7.6	8.8	no longer infective to mice					
In mechanical freezer at about −20° C. but not fluctuating	8.8	9.3	10.0	9.0	10.0	9.0	10.0	10.0*	—
In mechanical freezer at −20° C. until day 8 then subsequently in alcohol-dry ice mixture in Dewar flask	6.8	7.8	7.0	8.2	8.2	8.4	8.4	—	—
In alcohol-dry ice mixture after 2 days	—	—	—	8.2	8.2	8.4	7.8	8.8	8.0

* Only two of three mice inoculated died of toxoplasmosis.
— No trial on this day.

Influence of the concentration of glycerol and the diluent of the glycerol solution.

Another series of experiments was conducted in which the concentration of glycerol was varied over a wide range, and in which physiologic saline and distilled water were used as diluents. These experiments are summarized in Table 2 and the efficiency of preservation is estimated both on the basis of the time to death of inoculated mice and the reduction in infective titer of the suspension.

It is clear that survival of parasites is favored by low concentrations of glycerol; in fact, the lowest concentration employed, five percent by volume, showed the least reduction in infectivity as compared with the pre-freezing infectivity. Results were progressively poorer as the glycerol concentration was increased, and with 35 percent glycerol not all mice inoculated with undiluted material succumbed to toxoplasmosis.

Since the best results were obtained with the lowest glycerol concentrations used, experiments were conducted using slow freezing without glycerol. Survival of parasites could not be demonstrated.

The comparison of saline and water diluents is not good due to the fact that the concentrations used were not identical; however, on the basis of the data at hand, physiologic saline would be the choice of diluents.

Optimum technique as indicated by experiments so far.

Although future work may result in better definition of optimum conditions for

preservation, the following would appear to be the best procedure based upon the available results.

1. Prepare exudate as outlined in methods section.

2. Mix exudate with an equal quantity of 10 percent glycerol by volume in physiologic saline (giving a final dilution of five percent). Seal ampoules as described.

3. Freeze contents of ampoules by placing the ampoules in a test tube basket completely lined with a one-inch layer of absorbent cotton. Place in dry ice compartment of freezer for 24 to 48 hours then transfer the ampoules to a dry ice-alcohol mixture in a Dewar flask. The flask should be checked sufficiently frequently to insure that the dry ice does not become exhausted.

4. Thaw by immersing the ampoules in a 37° C. water bath until the contents are fluid. Open ampoule and inoculate clean mice with 0.1 ml. or more of the fluid.

This method has given consistently successful preservation up to at least 162 days after freezing and it is expected that longer preservation will be demonstrated when more time has elapsed. We are now using this technique for the storage of strains of *Toxoplasma* in our laboratory.

Caution should be used that the fragile ampoules are not broken; permanent labelling is a problem not yet completely solved by us.

TABLE 2.—*Influence of varying the proportion of glycerol and the medium in which the glycerol was dissolved on the freeze-preservation of* Toxoplasma

Diluent and final concentration of glycerol by volume	Titer for 50% infectivity			Mean survival days of mice given undiluted inocula
	Before freezing	After freezing	Reduction in titer	
Saline diluent				
5 percent	$10^{6.17}$	$10^{2.63}$	3.57	7.4
10 percent	$10^{6.17}$	$10^{2.17}$	4.03	7.4
15 percent	$10^{6.17}$	$10^{1.50}$	4.70	8.2
20 percent	$10^{6.17}$	$10^{1.50}$	4.70	8.2
25 percent	$10^{6.17}$	$10^{1.38}$	4.82	9.0
30 percent	$10^{6.17}$	$10^{1.38}$	4.82	9.5*
Water diluent				
7.3 percent	$10^{6.17}$	$10^{1.63}$	4.54	7.8

* Not all mice succumbed to toxoplasmosis.

DISCUSSION

The freezing of *T. gondii* provides a convenient means of preservation of strains of this organism. In our laboratory, we have had up to now the laborious task of maintaining by mouse passage five separate strains. The saving of time and materials possible through freezing has been considerable.

A further advantage of freezing is that it may be possible to maintain strains in an unchanged condition, not subject to the hazard of modification through continued animal passage. This is subject of course to the hypothesis, yet to be extensively tested, that freezing produces no modification.

Our efforts thus far have been directed toward the definition of optimal conditions. Although we have learned how some of the factors involved may be manipulated to give more efficient preservation, at best the present methods preserve

only a very small percentage of the organisms exposed to the freezing. Further work is necessary on the factors we have studied and other experiments are needed to determine the effect of other factors among which are the rate of thawing and the influence of the stage of development of the parasite.

SUMMARY

Efforts to preserve *Toxoplasma gondii* by slow freezing in glycerol solutions have been successful and survival of parasites in the frozen state has been demonstrated up to 209 days. Results of experiments varying the rate of freezing and the proportion of glycerol have indicated that very slow freezing using five to 10 percent glycerol is best. Even with the optimum methods, the initial loss of viability after freezing is great, but storage at below −70° C. results in little further loss of viability over the period tested. Rapid thawing for a few minutes in a water bath at 37° C. has been practiced.

It is suggested that the freezing technique should be a good method of preservation of strains without the cumbersome and dangerous necessity of frequent animal transfer, and this method of preservation is now being used in our laboratory. It is also suggested that the freezing method may allow the preservation of strains in their original state, unmodified by continuous rapid animal passage.

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RESEARCH NOTE

OPHIOTAENIA FILAROIDES LA RUE, 1909 (CESTODA: PROTEOCEPHALIDAE) REPORTED FROM UTAH SALAMANDERS

Metamorphosing specimens of *Amblystoma tigrinum* Green, collected at Dog Lake, Big Cottonwood Canyon, Salt Lake County, Utah, contained plerocercoids and young adults of a cestode which the author identified as *Ophiotaenia filaroides* La Rue, 1909. This specific diagnosis was confirmed by Dr. George R. La Rue of the Bureau of Animal Industry, U. S. Department of Agriculture, Beltsville, Maryland.

Dog Lake is a rather isolated, spring-fed lake lying at an elevation of approximately 8,000 feet on the western slopes of the Wasatch Mountains. The report of *Ophiotaenia filaroides* from salamanders from this lake is, as far as the author knows, the first reported occurrence of this cestode in Utah, and, for that matter, in this drainage area.

Specimens of this cestode were relaxed in 0.75% saline solution containing 0.4% Chlorotone (Parke-Davis). They were then fixed for two hours in hot (56° C) Bouin's fluid. They were stained with Delafield's iron haematoxylin and blued with ammonia alcohol.

Specimens were deposited by Dr. George R. La Rue in the Helminthological Collection of the U. S. National Museum, where they bear the number 55208. Additional specimens are in the zoological museum of the University of Utah.—JOHN C. FRANDSEN, *Department of Zoology, University of Utah, Salt Lake City.*

DEVELOPMENT AND MORPHOLOGY OF THE ONCOSPHERE OF *MESOCESTOIDES CORTI*, A TAPEWORM OF MAMMALS[1]

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Preliminary work on the oncosphere of this cestode has already been reported (Ogren, 1950, 1953). In the literature on the life cycle of this tapeworm, reviewed by Witenberg (1934), Webster (1949) and others, studies of embryonic development were not included. The writer realized that such study would more clearly define the taxonomic position of the Mesocestoididae and perhaps provide a further clue to the life cycle. Literature reviews of embryology occur in Hyman (1951) and Wardle and McLeod (1952). The writer wishes to acknowledge the encouragement and helpful advice of Dr. Lyell J. Thomas, during the course of this investigation.

HISTOLOGICAL METHODS

All specimens were obtained from raccoons, *Procyon lotor*, caught at Havana, Illinois, during the years 1949–1952. The specimens studied agreed with the morphology of *Mesocestoides variabilis* Mueller, 1927 which Voge (1955) considers to be a synonym of *M. corti* Hoeppli, 1925. Entire worms and gravid segments were killed, stretched and fixed in alcohol-formalin-acetic acid (AFA); Carnoy's; or in Zenker's. The best cytological material was obtained when worms were fixed fresh and active, without prolonged immersion in Ringer's solution. Carnoy's gave the best fixation, although AFA was also excellent. Serial sections were cut 10 microns thick. The entire worm was cut into small strips about one centimeter long. The correct order of segments was maintained by placing the pieces in serially numbered folders during dehydration and imbedding in tissue-mat. The same numbers were placed on the slides made from each piece. This procedure provided a complete picture of developmental stages taking place along the entire worm.

Cytoplasmic granules were stained with methyl green-pyronin, a reaction destroyed by ribonuclease (Ogren, 1952), demonstrating the presence of ribonucleic acid. Reaction with toluidine blue varied from dark-blue to purple and was considered to be a weak metachromatic reaction. Definite mitochondria were not stained successfully with aniline acid fuchsin following immersion in permanganate and oxalic acid baths (Lillie, 1954). Structures stained red by this procedure in the segments of *M. variabilis* studied by the writer were: basement membrane of cuticle, muscle fibers, cytoplasm, especially of subcuticular muscle cells, plastin cells and some parenchymal cells.

The periodic acid leucofuchsin (Schiff) procedure was employed in the form of Lillie's allochrome connective tissue method. A red to purple reaction usually indicates the presence of polysaccharides, mucopolysaccharides, glycoproteins or glycolipoids (Lillie, 1954, p. 121). Periodic acid-Schiff-positive materials in the segments were located in the cuticle, embryophore, oncosphere, parenchymal spaces

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[1] Part of this material was included in the dissertation submitted to the Graduate School, University of Illinois, Urbana, Illinois, in partial fulfillment for the degree of Doctor of Philosophy.

and around fibers. An orange reaction was given by muscle, subcuticular, plastin, flame and muscle cells. Blue-green reaction was given by transverse muscle fibers, basement membrane of cuticle, and cytoplasmic granules. Slides without albumin fixative gave the above reactions. Sections not placed in periodic acid for oxidation showed a faint pink coloration only in muscle bands. Compounds such as mucin and glycogen (Lillie, 1954, p. 357) were demonstrated by immersing sections in 0.5% sodium metabisulfite for 2 hours following the periodic acid bath. The Schiff-positive material was largely present in the parenchyma, embryophore, and oncosphere. The Castella reaction, using a 20-minute bath in 1% potassium permanganate in place of periodic acid to induce aldehyde formation, was weaker but localized as in the periodic acid-Schiff preparations. The Schiff reactions were abolished completely following a 30-minute bath in aqueous 5% phenylhydrazine hydrochloride before the leucofuchsin. Overnight immersion in leucofuchsin failed to restore this reaction. A 3-hour period of bromination, 1 ml bromine in 39 ml of carbon tetrachloride at 27° C, prevented reaction of all Schiff-positive substances in the segment and oncosphere. This reaction indicates the substance may be a polysaccharide similar to glycogen (Lillie, 1954, pp. 277, 311). Smyth (1947) indicated that glycogen was most abundant in the parenchyma of tapeworms. This is not a conclusive identification without enzyme digestion.

The ninhydrin-Schiff reaction (Lillie, 1954, p. 146), employing ninhydrin as the oxidizing agent, gives a positive reaction with muscle and collagen. Carnoy-fixed sections were immersed 23 hours in ninhydrin at 38°C, then placed in leucofuchsin. They showed intense pink coloration of cuticle, muscle bands, nuclei, oncosphere hook-region and plastin cells. The Millon reaction for proteins containing tyrosine (Lillie, 1954, p. 147) was weakly positive on Carnoy-fixed material in the cuticle, muscle and oncosphere.

The above histochemical reactions were employed primarily to examine the structure of the matrix in the oncosphere and more work is required to demonstrate specific organic compounds in the oncosphere. The following additional stains were used. Iron hematoxylin and fast green or acid fuchsin, van Gieson's connective tissue stain, Mallory triple stain, Feulgen technique, Unna's orcein, Lillie's metachromatic stain using Weigert's acid hematoxylin and safrinine O counterstain, basic fuchsin-picro-indigo carmine (Shumway).

PART I. EMBRYONIC DEVELOPMENT

Oogenesis

In immature segments plastin cells (primordial oocytes), with compact pyronin-stained cytoplasm, (Fig. 1) formed the primordium of the ovary. In slightly older segments, when the cirrus pouch primordium was prominent, the cells had enlarged (Fig. 2). Oocytes became granular with further enlargement (Figs. 3, 4), although regions of cytoplasm were compact. Cytoplasmic granules in these and older oocytes stained red with pyronin. Larger oocytes in nearly mature segments had small vesicles (Figs. 5, 6) perhaps associated with secretion. The granular cytoplasm increased with the increase in cell size (Figs. 7, 8) and included 1 or 2 vesicles. Mature oocytes in mature segments contained several large globules of darkly stained vitelline material (Fig. 9). The globules were present as lightly stained translucent spheres with a darker stained circumference, and as intensely stained

opaque spheres. The stain reactions of these spheres were similar to those of the vitelline substance. They stained blue-green with toluidine blue, orange with the allochrome periodic acid Schiff, purple with methyl green-pyronin, red with van Geison's and red with safranine O in the metachrome stain. The vitelline masses were still present when the oocyte was in the oviduct (Fig. 78). The masses were usually absent from fertilized oocytes in the uterine duct (Fig. 75). The globules eliminated from the cell were found in the uterine duct (Fig. 75) and similar material formed the shell around the oocyte (Fig. 71).

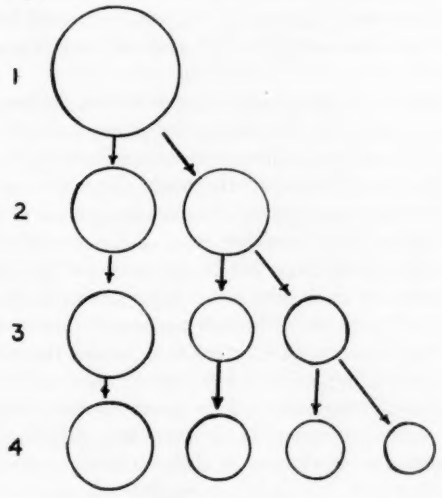
Oocytes commenced maturation divisions following entrance into the uterus. Oocytes in the uterine duct contained sperm (Fig. 75). The uterus, especially the posterior part, contained vitelline cells, vitelline globules and oocytes in various phases of meiosis (Fig. 71). The vitelline substance collected around the circumference of oocytes to form the capsule, which was not completed until after the morula stage (Figs. 73, 32). The vitelline substance at this time was colored by collagen stains. The first meiotic division took place (Figs. 18, 19) following entrance of the sperm. The second division reduced the number of chromosomes in the pronucleus (Fig. 20). Two polar bodies were clearly evident when both male and female pronuclei were mature. The sperm pronucleus enlarged (Figs. 10–14) until it was of a size equal to the female pronucleus. In some cases two sperm pronuclei (Figs. 16, 17, 20) were present in one oocyte. It is not known if these cells are capable of completing development. The fusion of pronuclei (Fig. 71) completed fertilization.

Cleavage

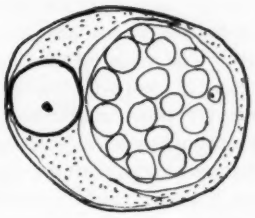
The zygote divided into 2 nearly equal blastomeres (Figs. 21, 22). This was not an immediate division since 2-cell stages were absent in a long series of proglottids. These 2 blastomeres, which for convenience were designated primary and secondary macromeres, were enclosed by the vitelline capsule. The polar bodies, adjacent to the blastomeres (Fig. 21) and an occasional intensely stained vitelline cell, were enclosed within the capsule. In older segments, various stages were packed together, indicating that the embryos did not develop synchronously in each segment. Division sequence appeared to be asynchronous in that blastomeres did not divide in unison. This was concluded by comparing the cell sizes present.

Early embryos appeared as irregular mixtures of cells of several sizes which made study very difficult. It was not known whether all embryos developed by the same cleavage pattern; however, the assumption was made that they did. A basic pattern was found as partial confirmation by comparing the average nuclear diameters from 10 typical embryos in the first 5 stages. This pattern was asynchronous in that the secondary macromere failed to divide until late in development and was excluded from the embryo and the primary macromere produced many cells, one group dividing more rapidly than the rest. The zygote nucleus measured 10 microns. The first cleavage produced 2 cells whose nuclei measured 6.8 microns. The 3-cell stage showed 2 nuclear sizes, a large nucleus (6.5 microns) represented the undivided secondary macromere, 2 smaller nuclei (4.5 microns) represented the division of the primary macromere. In the 4-cell stage nuclear sizes were found (6.4, 5.2, 4.0–4.5 microns) suggesting that a third cleavage had occurred represented by the 2 smaller cells. In the 5-cell condition four nuclear sizes were found (6.8, 5.6, 4.6, 3.6–3.8 microns) suggesting that a further division had occurred.

Microscopic studies of the cell lineage type should be made on living dividing embryos to establish the pattern experimentally. Although such studies could not be completed on sectioned material there was a definite pattern in the first 3 cleavage stages (Text-Figure 1), and a typical arrangement of the morula (Text-Figure 2).



TEXT-FIG. 1.



TEXT-FIG. 2.

All blastomeres contained numerous larger cytoplasmic granules, spread in their cytoplasm, that stained red with pyronin and gave a weak purple metachromatic reaction with toluidine blue. Three-cell stages, difficult to find, were of the form shown in Fig. 23. One blastomere was slightly larger than the other 2 nearly equal cells. Embryos with 4 blastomeres (Figs. 24, 25), contained cells of 3 different sizes: 1 large blastomere, the size of the secondary macromere, 2 small cells nearly equal in size, and a fourth cell often smaller than the others. Five-cell embryos similarly manifested inequality of blastomeres (Fig. 26). Divisions continued until in 6-, 7-, 8-cell stages the blastomeres tended to be more equal in size (Figs. 27–30). In the 10-cell embryo a close association of blastomeres was formed. However, 1 large blastomere the size of the secondary macromere was excluded from the morula, appeared to surround the morula, and in later development failed to take part in forming embryonic structure (Figs. 32–34, 42), but formed a thick collagenic membrane beneath the vitelline shell (Figs. 45, 76).

Cellular Differentiation in the Embryo

The morula (Fig. 32) developed into a spherical embryo (Figs. 33–42). The following evidences of cellular differentiation were found which resulted in the formation of the hexacanth, or oncosphere. 1) Cells at the surface (Fig. 33) multiplied, became squamous (Figs. 34, 41), separated from the inner cell mass (Fig. 35), and formed a cellular membrane (Fig. 42), the embryophore, around the oncosphere. 2) Poorly stained cells with few granules around their nuclei and with indistinct cell membranes, formed the mesenchyme of the oncosphere (Figs. 36, 37), especially in the hook-region. 3) Hooks, derived from oncoblasts, appeared at the

anterior portion (Fig. 42). 4) Large cells with intensely stained pyronin granules and definite cell boundaries, were located at the presumptive scolex region, the embryonic posterior (Figs. 37, 38). Cells in this region later became plastin cells typical of older oncospheres. The embryo consisted of a hexacanth inner cell mass or oncosphere, enclosed by the embryophore, a thin squamous epithelium-like membrane derived from small surface blastomeres. Nuclei and granules derived from the secondary macromere were identified between the shell and the embryophore (Fig. 42).

The epidermal cells that formed the embryophore were squamous and frequently only a few were seen in one visual plane (Fig. 37), or in the pattern shown by Fig. 40. The nuclei of developing embryophore cells contained prominent nucleoli. A region of compact cytoplasm (Fig. 35) surrounded the nuclei. The remainder of the cytoplasm was granular. The compact cytoplasm and granules gave a prominent pyronin coloration, colored purple with toluidine blue, and red with the allochrome procedure. In later stages of development the cytoplasmic granules were less numerous, being confined more to the inside of the thin membrane (Figs. 35, 43, 45). Nuclei and their remnants were more difficult to demonstrate at this (Fig. 48). In its final condition the embryophore, a thin membrane around the oncosphere, was closely applied to the shell (Fig. 45). Embryophore formation appeared to represent an early form of secretory activity in which metachromatic granular secretion was associated with the formation of protective membranes. Moreover, these changes may indicate the senescence of differentiated epidermal cells.

PART II: MORPHOLOGY OF THE ONCOSPHERE

The oncosphere was composed of granular cells of various sizes and stainability. A thin cuticle covering the surface gave a collagen reaction. Two groups of cells were recognized in young oncospheres (Fig. 37). Small cells, confined largely to the region of the hooks, were identifiable as "somatic cells" of Wisniewski (1930). Large cells, with prominently stained cytoplasmic granules, located in the region opposite the hooks, were identifiable as "germinative cells." These cells were distinguishable on the basis of size, location and stainability.

The cells forming the mesenchyme of the hook-region had small nuclei, usually with small eccentric nucleoli, or unrecognizable nucleoli, and in young oncospheres contained many pyronin granules (Figs. 35–37). Cell boundaries were difficult to observe and the hook-region presented a syncytial appearance. There was a poorly stained matrix between the cells. As the oncospheres became older, the granules in the hook-region decreased and the pyronin reaction nearly disappeared (Figs. 43, 46). Nuclei became difficult to demonstrate in the hook-region, by the Feulgen technique (Fig. 48), or with methyl green-pyronin (Fig. 46). The abundant cells in the hook-region were replaced by a protoplasmic matrix containing very few nuclei (Fig. 45). This matrix enclosed the cell mass opposite the hooks and infiltrated between the cells. The matrix was stained best by collagen stains: Aniline blue, methyl blue, fuchsin, fast green. Good differentiation was obtained with safranine O and Weigert's hematoxylin (Fig. 45), aniline blue orange G, or aniline acid fuchsin in the mitochondrial procedure. With the periodic acid-leucofuchsin reaction, followed by Weigert's acid hematoxylin a possible glycoprotein or polysaccharide substance in the matrix was colored red and the compact

plastin cells were black (Fig. 66). A similar parenchymal substance in the proglottid also gave this reaction. With the allochrome procedure, the matrix stained red, compact cells were orange (Fig. 47, 49). In some specimens the Schiff-positive material was deposited on the subcuticular network (Fig. 72). This cortical network was also faintly stained blue-green in Feulgen preparations counterstained with nile blue sulfate, or aniline blue. The exact manner in which the matrix developed was not learned. Small globules of Schiff-positive substance were observed (Fig. 41), often very abundant between the cells before hooks appeared, but infrequent in oncospheres. These globules stained red with methyl green-pyronin. There was a gradual accumulation of Schiff-positive material (glycogen?) in the embryo, becoming abundant in the oldest oncospheres. In fact so much was present that the cells were obscured in the allochrome procedure.

The largest cells of the oncosphere, with large pyronin granules, were found in the region opposite the hooks (Fig. 37). The large nucleus had a prominent nucleolus. By the time hooks were fully formed, cells in this region were still prominent, but were smaller and more numerous (Figs. 38, 42). Older oncospheres contained a new type of cell (Fig. 42) with compact cytoplasm manifesting an intense pyronin reaction. This new cell type completely replaced the granular cells (Fig. 46). In this cell, the plastin cell of Vogel (1930), the granules were packed closely together, giving a compact appearance to the cytoplasm. In subsequent development the numbers of these plastin cells increased (Fig. 43) until in the oldest oncospheres they were the predominant cell. An exact source of the plastin cells was difficult to discover. It is logical to expect they were produced by mitosis of similar cells multiplying forward into the hook-region. However, mitotic figures were not observed to account for this proliferation. Moreover, if one cell could form compact cytoplasm, other cells could also. A second view is more in line with microscopic studies. The plastin cells may be derived from mesenchyme cells of the oncosphere, whose granular cytoplasm becomes compact. The beginning of this process can be seen in Figure 42. This view accounts for the disappearance of nuclei in the hook-region by the conversion of these cells into plastin cells. It also accounts for the extension of plastin cells into the hook-region through the transformation of cells nearest the plastin cell mass.

The kind of cells present in the oncosphere depended on the stage of development. Young oncospheres (Fig. 42) contained cells with varied amounts of granular and compact cytoplasm around the nuclei. Older oncospheres (Fig. 43) contained largely plastin cells with a few poorly stained mesenchyme cells. The oldest oncospheres in segments from the stools contained plastin cells as the only stainable cell type. The transformation of granular cells into compact plastin cells best explained these observations. The real difference, therefore, between somatic and germinative cells breaks down. Most of the cells in the oncosphere became plastin cells and were thus potentially germinative in function.

The compact cytoplasm of plastin cells was intensely stained. The extent of the cytoplasm was shown by the use of the Feulgen technique (Fig. 48), as compared to control methyl green-pyronin preparations (Fig. 46). The pyronin material in the cytoplasm was removed by ribonuclease and could not be stained afterwards with pyronin. The cytoplasm was stained red to red-orange with neutral red *in vivo*, dark blue to purple with toluidine blue, red with Mallory triple stain, orange

with the allochrome procedure, red with aniline acid fuchsin for mitochondria. Similar reactions were given by muscle, subcuticular and uterine cells.

Since the plastin cell region became the scolex region of the metacestode and adult, this part of the oncosphere was called the presumptive scolex region. Plastin cells, first described by Vogel (1930) in the coracidium of *Dibothriocephalus latus,* were identified on the basis of the pyronin reaction of the "plastin" or compact cytoplasm. He considered these cells to be embryonic cells capable of forming all the structures of the metacestode and adult. The writer has observed the differentiation of plastin cells in the adult strobila of *Mesocestoides* into oocytes, subcuticular cells, uterine cells, gonads and muscle.

The Hooks were the most noticeable structures, exclusive of plastin cells, present in oncospheres. There were 6 hooks each consisted of a curved blade, and a straight shank (Fig. 44). The junction of blade and shank was the collar and represented the attachment of the hook to the cuticle. An arbitrary orientation of the oncosphere was possible with reference to the hooks. The surface toward which the medial hooks pointed was called ventral. Since oncospheres moved slowly with the hook region forward, this region was called anterior. A lateral view (Fig. 60) indicated the medial 2 hooks were long (17 microns) and slender. A ventral view of the oncosphere (Fig. 61) indicated that each lateral pair of hooks (11 microns long) was situated on the same plane. However, the anterior-dorsal hook in each pair tended to be more slender and sometimes not as long (Figs. 44, 57). A frontal view (Figs. 59, 65) of the oncosphere showed the sectioned shanks of the hooks, demonstrating the dorsal-lateral shanks to be of smaller diameter. Because of differences in development this may not always be the case. The collar was attached to the cuticle and to a subcuticle cell (Figs. 56, 57). At this attachment the collar had a sharp barb. The shapes of hooks in certain cases were made diagrammatic such as in Figs. 50, 51, 62. However, in most cases they were based on camera lucida tracings. In some cases only part of the hook was visible, or it was traced rapidly in a living specimen. An attempt to present true size and shape relationships in the specimens is made in Figs. 44, 56–60. The blades were always clearly seen but the terminal end of the shank, embedded in matrix, was obscured and varied with stage of development. Fiber connections to the medullary end, more certain than those of Fig. 58, were difficult to demonstrate.

Study of developing oncospheres indicated hooks were secretions from oncoblasts and began development during embryophore formation (Fig. 42), actually in a stage earlier than that figured. The curved blade was secreted first from the granule-filled cells as a sharp projection above the cuticle (Fig. 44). The shank was formed next beginning at the collar (Fig. 44). Thus the shank of a young lateral hook presented a slender and somewhat triangular appearance with a very narrow terminal end. The terminal end of the shank would be the youngest part of a hook, the blade the oldest. The hooks were usually difficult to stain when fully formed. Recently formed blades in Carnoy-fixed material stained red with pyronin, purple with toluidine blue, red with safranine O. The hooks were stained a bright red with aniline acid fuchsin, and red in the Shumway stain. Good contrast was also obtained with the metachrome stain.

Embryonic Musculature

Search for muscle fibers was difficult and results did not agree with the writer's

original notions. Contractile fibers in the adult stained for muscle and collagen. In the tetrathyridium, muscle stained for collagen. It was concluded that proper staining would reveal muscle in the oncosphere. Iron hematoxylin and fast green preparations gave best results. In the most gravid segments from feces, oncospheres contained definite muscle bands (Figs. 67, 68) located in the hook-region. Such bands were confirmed only with Mallory triple stain. Present in these preparations were a few delicate fibers going forward on the hook-region. Occasional circular bands were also found. In young oncospheres stained with pyronin fibers were observed going forward from the granular cells in the posterior region (Fig. 38). The intercellular matrix in the oldest oncospheres described was demonstrated with collagen stains. In the subcuticular region this matrix was a network.

In living oncospheres observation of muscle was difficult. The entire oncosphere was contractile. The subcuticular matrix was associated with plastin cells which showed contractile properties. Grey, intercellular, protoplasmic substance (Figs. 54, 69), appeared to contract beneath the cuticle during movements. Comparison of Figures 54 and 43 indicated that the plastin cells formed the small contractile "knob" at the posterior end of the oncosphere. A subcuticular network of contractile nature was observed associated with the hooks (Fig. 64), and the matrix of the hook-region. Also, delicate circular contractile fibers (Fig. 55) were observed in the subcuticular region, associated with the medial hooks. These fibers shortened by becoming more wavy (Fig. 70). This was also true of isolated fibers of adult muscle. In cestode muscle the myoblast is associated by delicate processes with the abundant contractile fibers.

Deep focusing into the body during movements indicated that the matrix had contractile properties. The outline of contractile substance (Figs. 62, 63) coincided with the outline of the plastin cells. Open spaces in the figures contained cells. Fibers were associated with the free end of the hook (Fig. 74), and extended into the matrix of the plastin cell mass. It was not proved that these fibers shortened, although they pulled the hooks into the body. The central medullary region of the oncosphere was protoplasmic, and movements of extension seemed to originate in this contractile mass (Fig. 62, 63). The intensity of collagen stain was greatest at the base of the medial hooks.

Living Oncosphere

Gravid segments were recovered from the feces, and the paruterine organ was teased out in mammalian Ringer's solution, placed in solution on a slide and broken open. The white, sticky, gelatinous contents contained numerous tightly packed embryos. The shell of the embryo was irregular (Fig. 76) and tended to stick to other shells due to adhesive properties of the vitelline substance. With a small pipette, embryos were transferred to a slide and examined under oil immersion.

Living unstained "eggs" had the appearance of Figure 74. A thick, many layered, transparent, rigid membrane, enclosed the oncosphere. Beneath this were small groups of irregular accretions resting upon the thin transparent embryophore. The oncosphere had a delicate cuticle from which delicate connections went to the embyrophore. These connections varied with the embryo and appeared to represent accumulations of adhesive material secreted through the cuticle. They became more evident when the embryo was placed in hypertonic media. Compact plastin cells could be seen beneath the cuticle.

A crude digestive extract obtained from crushed *Cypris* sp. permitted the release of oncospheres alive from their membranes. By this means the capsule was softened and the adhesive connections of the embryophore were destroyed, allowing the oncosphere to move freely and work its way out. During this procedure, the embryophore could be distinguished from the shell capsule, since it was frequently pulled out with the oncosphere (Figs. 50, 51). Oncospheres released from the shell frequently carried along with them 1 or more thin transparent vesicles of adhesive material, believed to be derived by limited diffusion of adhesive material through the thin cuticle (Figs. 52, 53). Granular secretion stained yellow with neutral red and showed Brownian movement in the hook region of some oncospheres (Figs. 52, 67). These granules are not to be confused with the granular "penetration glands" of Reid (1948) or with the granular areas described by Ogren (1955). The small granules in the *Mesocestoides* oncospheres gave similar reactions with neutral red, but showed no real pattern, were seen best in dying oncospheres, and appeared to represent accumulated secretion.

Flame cells were never observed in these oncospheres. It is believed that they do not develop until the metacestode stage. Flame cells were not seen in sectioned oncospheres although they were readily recognized in the parenchyma of the segment.

Oncospheres with their capsules, upon release from the paruterine organ, did not move at first. There was a period of a few minutes before the rhythmic movements began. These then continued until death. Movements were manifested as extensions followed by relaxation or retraction. Extension (Figs. 53, 62) was accomplished by a wave of constriction beginning anteriorly and ending posteriorly often producing here a "knob" (Figs. 54, 74). By this movement the lateral hooks were pulled sideward and their blades were extended. The medial hook blades were extended forward, slightly laterally and scraped downward on the substrate. In retraction (Figs. 52, 55) the body enlarged and the hooks were pulled back to the medial resting position, parallel to each other. Movement was not always uniform. The oncosphere presented the appearance of struggling to move regularly. The entire movement seemed to concentrate on thrusting the medial hooks forward. Movements of the lateral hooks was secondary to the thrust of the medial hooks. Occasionally the hooks, especially the lateral ones, became asynchronous and made local movements. The conclusion of observations was that the regular extensions of the oncosphere and subcuticular contractions caused the hooks to move. Witenberg (1934) has previously described oncosphere movements of *Mesocestoides*.

Living oncospheres stained with neutral red continued to move for several hours and provided nicely colored preparations. Plastin cells were the primary cells stained in oncospheres from gravid segments (Fig. 76). A few nuclei of hook-region cells could be seen by careful focusing. The compact cytoplasm of plastin cells stained red, sometimes with an orange tint. In some oncospheres the plastin cells contained pink granules. The oncospheres showed differences in number of plastin cells (76, 77). Oncospheres containing mostly plastin cells had only a few nuclei in the hook-region. The stained nucleolus of a cell presented the appearance of a large granule dancing in a clear vesicle (Fig. 69).

PART III: DISCUSSION

Sufficient information is not yet available to make extensive generalizations.

However, there are several points learned from this study of *M. variabilis* that may provide the basis for comparison with various cestode embryogenies.

1). The nature of the oocyte, whether or not it contains vitelline globules. 2). The pattern of first divisions and whether or not all blastomeres form the embryo; i.e., is the secondary macromere excluded from the morula? 3). Appearance of the morula stage including egg capsule. 4). Whether or not the embryophore is formed and detached. 5). Whether the embryophore is membranous or ciliated. 6). Size and morphology of hooks. 7). Presence or absence of flame cells. 8). Presence or absence of granular gland regions. 9). Abundance of plastin cells. 10). General appearance of the oncosphere.

Identification of germ layers is not possible with certainty at this time. Presumably this could be settled by *in vitro* studies of cell lineage and cleavage patterns. However, Child (1941: p. 554) stated that the rule of strict cell homologies does not hold. Identification of germ layers in the final analysis is made on the basis of the type of tissue formed, i.e., the ultimate fate of the group of embryonic cells (Hyman, 1940: p. 230). Moreover, in the Platyhelminthes, "layers" are not present. In *M. variabilis* the oncosphere was composed of plastin or embryonic mesenchymatous cells. Hyman (1951) considered this cell mass of an oncosphere to be "mesentoderm." Adult structures formed by these cells are muscle, parenchyma, gonads, ducts, secretory cells. All these are ordinarily considered to be mesodermal derivatives. Recognizable endodermal derivatives were absent. The body covering of *M. variabilis* is not considered by the writer to be a digestive epithelium and, therefore, not endodermal. This conflicts with certain recent theoretical views published (Clarke, 1954 and Wardle and McLeod, 1952). In the present state of our knowledge of tapeworm embryology all that can be said is that the tapeworm strobila is predominantly "mesodermal." The plastin cells, their formation and cellular differentiation, need further study since they form adult tissues and organs.

The study of tapeworm development involves many problems of cellular biology such as contractility, secretion, cellular differentiation. Knowledge so far indicates that the abundant cytoplasmic granules, probably associated with mitochondria, are important for secretion and merit further study. Cultivation of embryonic cells in tissue culture and isolation of these granules would help acquire this knowledge. Important secretory products formed are hooks, membranes, cuticle, "calcareous" or phosphate bodies.

SUMMARY

Oocytes are derived from plastin cells (primordial oocytes) matured in the ovary. As oocytes grow, their cytoplasm becomes granular. Mature oocytes contain large spheres of vitelline substance. Meiosis occurs with the sperm nucleus present while oocytes are in the uterus. The zygote, enclosed by a capsule of vitelline material, divides into 2 nearly equal blastomeres. One blastomere divides slowly, is excluded from the morula and forms no part of the embryo. The other blastomere divides into cells which cleave at various rates to form a morula of small cells. An embryonic epidermis, the embryophore, develops from small, squamous, granule-filled cells at the surface of the embryo and separated from the inner wall mass. The latter develops 6 hooks and becomes the oncosphere. The morphology and development of hooks were considered briefly. The granules and membrane of

the embryophore are metachromatic and give a positive reaction with the periodic acid-Schiff procedure suggesting the presence of glycoprotein or mucopoylsaccharide substances. The parenchyma is composed of small cells giving a weak pyronin reaction. Typical plastin or germinative cells differentiate from granular cells and concentrate in the presumptive scolex region of the oncosphere. The morphology of sectioned oncospheres is described. The behavior and visible structure of living oncospheres are presented. Flame cells are absent. Granular areas ("penetration glands") as found in *Hymenolepis* are absent. The basic knowledge necessary for comparative embryonic studies is suggested. The tapeworm tissues are considered to be predominantly "mesodermal" in morphology and, probably, in origin.

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Description of Figures

All drawings made with the aid of camera lucida except Figures 64 and 70. All Figures except 50, 51 are oil immersion, 950× and have the same scale as on plate I. One scale division equals 10 microns. Abbreviations for stains used: methyl green-pyronin (MP), hematoxylin-van Gieson (HVG), allochrome periodic acid-leucofuchsin (PAL), neutral red (NR), toluidine blue (TB), aniline acid fuchsin (AAF), iron hematoxylin—fast green (HG), Lillie's metachromatic hematoxylin and safranine-O (MHS).

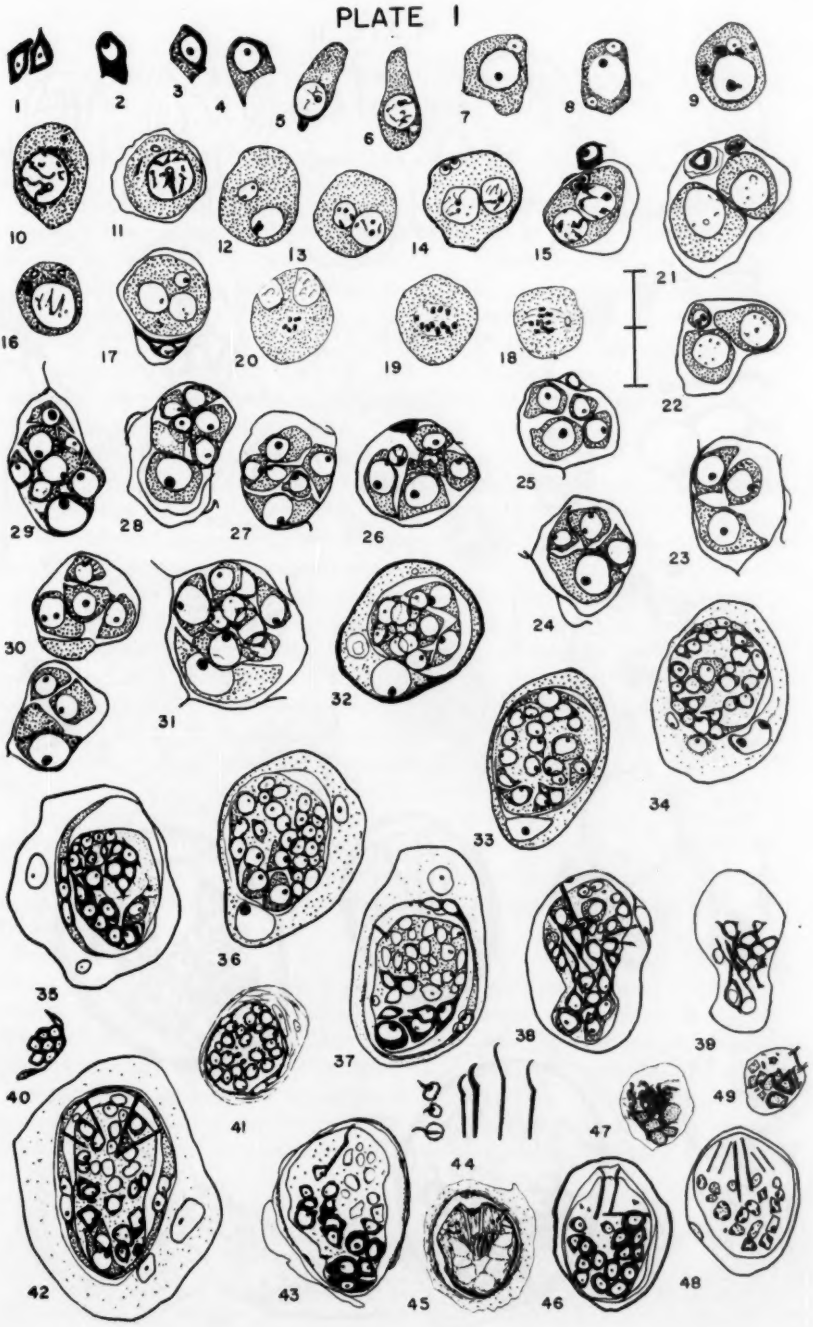
PLATE I

1. Plastin cell, primordial oocytes (MP).
2. Enlarging oocyte, immature segment (MP).
3. Enlarging oocyte, granular and compact cytoplasm (MP).
4. Enlarging oocyte, granular and compact cytoplasm (MP).
5. Enlarging oocyte, nearly mature segment, vesicle (PAL).
6. Enlarging oocyte, blue granular cytoplasm, vesicle and vitelline globule, same immature ovary as Fig. 5 (PAL).
7. Nearly mature oocyte, densely packed, purple granules (TB).
8. Nearly mature oocyte, 2 vesicles, purple granules (TB).
9. Mature oocyte in ovary, faint purple granules, blue green vitelline masses, one is more translucent (TB).
10. Mature oocyte with sperm, in uterine duct (HVG).
11. Oocyte in uterus, male pronucleus enlarging (MHS).
12. Mature oocyte in uterus, enlargement of male pronucleus (MHS).
13. Mature oocyte in uterus, enlargement of male pronucleus (MHS).
14. Oocyte in uterus, male and female pronuclei, polar bodies (HVG).
15. Same as 14 with vitelline cell (HVG).
16. Oocyte in uterus, two sperm pronuclei and vitelline globules (MHS).
17. Oocyte in uterus, two male pronuclei, one female (MHS).
18. Oocyte in uterus, female nucleus undergoing first meiotic division (MHS).
19. Oocyte, first maturation chromosomes (MHS).
20. Oocyte in uterus, two male pronuclei, female nucleus has completed second meiotic division (MHS).
21. Two-cell stage, first cleavage vitelline cell, polar bodies (MP).
22. Two-cell stage (MP).
23. Three-cell stage (MP).
24. Four-cell stage (MP).
25. Four-cell stage (MP).
26. Five-cell stage, one vitelline nucleus (MP).
27. Six-cell stage (MP).
28. Seven-cell stage (MP).
29. Eight-cell stage (MP).
30. Seven-cell stage, upper and lower planes drawn separately (MP).
31. Ten-cell stage, secondary macromere at bottom (MP).
32. Morula, granular cytoplasm of macromere surrounding the embryo (MP).
33. Beginning of embryophore (MP).
34. Solid embryo, distinction between inner cell mass, squamous epidermal cells of embryophore and nuclei from divisions of secondary macromere; large and small cells of inner cell mass (MP).
35. Complete separation of embryophore from inner cell mass (MP).
36. Advanced morula showing different cell sizes, embryophore beginning to form (MP).
37. Section of early embryo showing cellular types in oncosphere and detached embryophore (MP).
38. Embryo, membranous embryophore, large granular cells (MP).
39. Surface view of subcuticular matrix stained red (MP).
40. Arrangement of cells in complete embryophore, cell borders stained red (PAL).
41. Embryophore and globules stained red, cell granules, blue green (PAL).
42. Hexacanth embryo, detached embryophore, four-cell types in oncosphere (MP).
43. Advanced embryo in which the oncosphere contains plastin cells and a few poorly stained cells in hook-region. Embryophore is membranous (MP).
44. Hooks, figures at left are stages in development (MHS).
45. Embryo, stained to show matrix. Plastin cells were stained black in the outlined spaces. Matrix was pale pink, dark lines near hooks were fibers. The granules of shell were blue, the thick zone of fibers around embryophore was red (MHS).
46. Embryo, as 45, from most gravid segments in feces, plastin cells are the predominant cell, compact cytoplasm was red (MP).
47. Medullary intercellular substance was red, granular plastin cells were orange (PAL).
48. Embryo, as 46, Feulgen reaction to show distribution of nuclei and extent of compact cytoplasm.
49. Section of oncosphere showing plastin cells. The black part of the cell stained red, granular material stained yellow-orange (PAL).

PLATE II

50. Oncosphere escaping from shell, embryophore still attached, 440×.
51. Living oncosphere, out of shell, embryophore free, 440×.
52. Living oncosphere, free of membranes, clear side vesicle.
53. Living oncosphere, with vesicle.
54. Living extended oncosphere, within shell, dark lines represent lines of contracted material.
55. Living retracted oncosphere, contractile fibers in cortex associated with hooks.
56. Lateral hook attached by collar to contractile cell (AAF).
57. Lateral hooks showing different relative size (AAF).
58. Lateral hook showing nature of medullary attachment in the protoplasmic matrix. Dark lines represent fibers (AAF).
59. Oncosphere, frontal view, position and size of hooks (AAF).
60. Lateral view, difference in size and position between medial and lateral hooks. Dark hooks are nearest observer (AAF).
61. Ventral view of oncosphere showing hooks. Shaded matrix was pink, plastin cell region in outline was red, hooks red (AAF).
62. Living, extended oncosphere. Hooks diagrammatic for shape to show position. Dark shading represents contractile material between plastin cells.
63. Living oncosphere, as 62.
64. Contractile network in cortex associated with hooks, freehand.
65. Oncosphere, frontal view, showing pink stained matrix around hooks (AAF).
66. Oncosphere showing red stained substance in matrix (PAL).
67. Oncosphere, from most gravid segments, black muscle bands in cortex over medial hooks (HG).
68. Oncosphere, black stained muscle bands between hooks (HG).
69. Living oncosphere, oblique view, contractile substance between plastin cells is black in Figure. Nucleus with a red stained nucleolus is shown with its plastin cell (NR).
70. Diagrammatic representation of muscle fiber contraction, left shortening, right elongation.
71. View of oocytes following entrance into uterus. Black vitelline material and shell membrane was stained red (HVG).
72. Oncosphere, subcuticular matrix stained red (PAL).
73. Sectioned embryos of morula stage as they appear in uterus (HVG).
74. Living, unstained, embryo, showing thick fibrous shell, fibers attached to hooks, and protoplasmic fibrils from cuticle to embryophore.
75. Section of uterine duct showing oocyte with sperm nucleus, vitelline cells and vitelline globules (HG).
76. Living oncosphere, gravid segments, red stained plastin cells, orange stained hook-region nuclei (NR).
77. Living oncosphere, same segment as 76, but with more plastin cells (NR).
78. Oocyte in oviduct, oocapt on right, wall of paruterine organ at top (HVG)

PLATE I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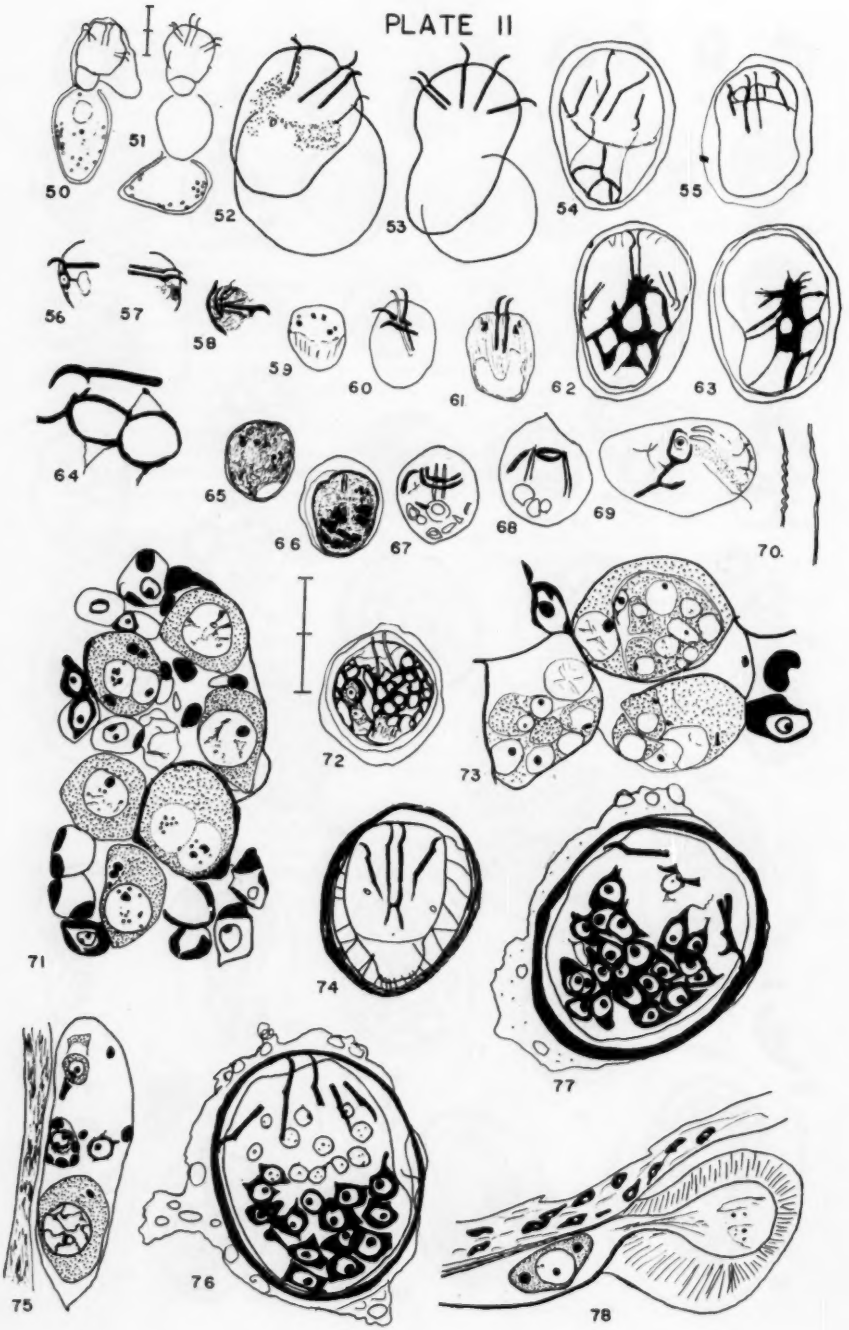
PLATE II
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LARVAL TREMATODE INFECTIONS IN SNAILS OF DIFFERENT SIZES*

A. W. Mathies, Jr.[1] and W. W. Cort

When a species of trematode utilizes snail intermediate hosts of different sizes even casual observations indicate that larger numbers of germinal sacs develop in the larger snails. In a few cases counts of the secondary germinal sacs have shown striking differences in numbers. Wisniewski (1937) made counts of the daughter rediae in infections of *Parafasciolopsis fasciolaemorpha* in *Coretus corneus*. He found that in 5 snails with a shell diameter from 12 to 16 mm the numbers of daughter rediae varied from 670 to 1800 with an average of 1057, while in 6 larger snails of the same species with shell diameters from 22 to 32 mm the numbers varied from 1700 to 8000 with an average of 4217.

This difference in numbers of daughter rediae may be even greater when a species of trematode infects two or more species of snail hosts that are considerably different in size. Cort, Ameel and Van der Woude (1948) reported in 9 specimens of *Helisoma antrosum* with shell diameters from 10 to 12 mm, infected with *Echinostoma revolutum*, the numbers of daughter rediae varied from 175 to 540 with an average of 360, while in 7 specimens of *Helisoma trivolvis*, with shell diameters of approximately 25 mm, infected with this same echinostome, the numbers varied from 558 to 3960 with an average of 1724. The same relation was also found by these workers in infections of a psilostome, *Ribeiroia ondatrae*. In 8 specimens of *H. antrosum*, with shell diameters of 10 to 12 mm, the numbers of daughter rediae varied from 260 to 606 with an average of 434, while in two specimens of *H. trivolvis*, with shell diameters of approximately 25 mm, the numbers were 1432 and 2677. Similar differences were found in infections of *Clinostomum marginatum* (*cf.* Cort, Ameel and Van der Woude, 1950). In 11 specimens of *Helisoma campanulatum*, with shell diameters of about one-half inch, the counts of daughter rediae ranged from 1075 to 3131 with an average of 2025, while the counts in two specimens of *H. trivolvis*, in which the shell diameter was approximately one inch, were 4002 and 4072.

During the summer of 1955 we compared the sizes of mature infections of *R. ondatrae* and two echinostome species, *Petasiger nitidis* and *Echinoparyphium recurvatum* in snail intermediate hosts of different sizes. The counts of daughter rediae were made after they had been teased out from the digestive glands of the snails and killed in hot 10 percent formalin. The size of the rediae is quite variable in a single infection, and they are larger in old infections. However, an attempt was made, by measuring the length of 25 of the largest rediae in each infection studied, to determine if the size of the rediae was influenced by the size of the snail host.

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In can be seen from Table I that the counts of rediae of *R. ondatrae* were almost 10 times as large in the one infected specimen of *H. trivolvis* and as in the three specimens of *H. campanulatum* in which the shell diameter was less than half as great. Also, the lengths of the largest rediae were slightly greater in the large snail than in the smaller ones.

In the infections with *P. nitidis* the numbers of daughter rediae were from 9 to 15 times greater in the larger specimens of *H. trivolvis* as compared with the smaller *H. campanulatum*. In these infections, however, the average length of the largest rediae was slightly greater in the smaller snails.

With *E. recurvatum*, a common echinostome of the Douglas Lake region, we were able to make studies on mature infections in snail hosts ranging in size from adult *Fossaria abrussa* and small juveniles of *Physa parkeri* and *Stagnicola emarginata* to large adults of *Bulimnea megasoma*. In all these infections there appeared to be no correlation between the lengths of the daughter rediae and the size of the snail hosts. Most striking perhaps are the enormous numbers of rediae that developed in the digestive glands of the infected specimens of *B. megasoma*. In the infections in juveniles and adults of *P. parkeri* the numbers of rediae appeared to be definitely related to the size of the snails. In the 5 adults of this species the numbers of rediae varied from 741 to 1444 with an average of 985 while in the 4 smaller juveniles the counts ranged from 422 to 738 with an average of 579. The numbers of rediae of this species were the smallest in small juveniles of *S. emarginata* and in the single infected adult of *F. abrussa*. The infected adults of *S. emarginata* were unusually small snails so that comparison between juveniles

TABLE I.—*Number and size of daughter rediae in infections in snail hosts of different sizes*

Species	Snail host	Shell size in mm	Count of daughter rediae	Length in mm of daughter rediae	
				Range	Average
Ribeiroia ondatrae (Price, 1931) (cf. Beaver, 1939a, and Price, 1942)	*Helisoma trivolvis*	24 × 20	2,163	1.5–2.9	1.9
	H. campanulatum	11 × 8	252	1.3–2.3	1.6
	" "	11 × 8.5	217	1.3–2.2	1.5
	" "	12 × 8.5	362	1.3–1.6	1.5
Petasiger nitidis Linton, 1928 (cf. Beaver, 1939b)	*H. trivolvis*	22 × 18	5,841	0.8–1.3	1.0
	" "	20 × 16	5,079	0.8–1.4	1.1
	" "	22 × 22	3,523	0.9–1.3	1.1
	H. campanulatum	10 × 8.5	381	1.1–1.5	1.2
	" "	15 × 12	395	1.3–2.5	1.7
Echinoparyphium recurvatum (Linstow, 1873) Lühe, 1909 (cf. Harper, 1929)	*Bulimnea megasoma*	50 × 30	5,090	1.2–1.5	1.3
	" "	46 × 27	4,129	1.1–1.4	1.2
	" "	40 × 21	3,677	1.1–1.5	1.3
	" "	30 × 17	1,130	1.4–2.1	1.7
	Physa parkeri (adult)	21 × 16	770	1.0–1.3	1.1
	" " "	20 × 17	1,171	1.1–1.9	1.3
	" " "	16 × 12	741	0.5–1.2	0.9
	" " "	16 × 10	798	0.8–1.5	1.1
	" " "	17 × 13	1,444	1.2–2.1	1.5
	P. parkeri (juv.)	10 × 7	568	1.1–1.9	1.3
	" " "	12 × 8	586	1.1–1.9	1.3
	" " "	10.5 × 7.5	423	1.1–1.9	1.2
	" " "	15 × 11	738	1.6–2.7	1.9
	Stagnicola emarginata (adult)	13 × 10	520	1.3–1.9	1.4
	" " "	12 × 8	246	1.1–1.8	1.3
	S. emarginata (juv.)	17 × 11	252	0.8–1.4	1.1
	" " "	16 × 9	240	1.0–2.5	1.7*
	" " "	12 × 7	179	1.1–1.6	1.2
	" " "	11 × 6	183	1.1–1.7	1.3
	" " "	11 × 6	114	1.1–1.4	1.2
	" " "	10 × 6	224	0.5–1.1	0.7
	" " "	10 × 6	155	0.6–1.2	0.8
	Fossaria abrussa	11 × 4.5	130		

* Only 20 rediae measured; in all other cases 25 of the largest rediae were measured.

and adults is not significant. Only one infection with *E. recurvatum* was found in *F. abrussa*. The number of daughter rediae in this infection was next to the smallest found in any of the snails. Its digestive gland was probably the smallest of any of the snails in the series, since juveniles of *P. parkeri* and *S. emarginata* with comparable shell lengths had greater widths. It is interesting to note that the number of daughter rediae in the largest specimen of *B. megasoma* was almost 40 times as great as in *F. abrussa*.

In all these hosts the incidence of infection was quite low. It seems probable, therefore, that there were very few, if any, multiple infections in the series. All the infections studied appeared quite normal and all were giving off mature cercariae. The snail hosts all seemed to be in good condition and were quite active. In none of them did the digestive glands appear to be severely injured and the rediae did not seem to be any more crowded in the smaller snails than in the larger ones. It seems evident from the counts of the different infections that there was a definite relation between the size of the snails and the numbers of rediae that developed in them. This is true both in juveniles and adults of the same species and in snails of different species that differ in size. Also there is no evidence from the measurements of the rediae that they grow to larger size in the larger hosts. Since no mother sporocysts or mother rediae were found in any of these infections there is no way of knowing whether the smaller number of daughter rediae in the smaller snails was due to the production of smaller numbers of mother rediae by the mother sporocysts or to a reduction in production of daughter rediae by the mother rediae. Since the differences in numbers are so great, it seems probable that both factors operate. The mechanism by which the small size of the snails limits the production of daughter rediae is difficult to understand. In any case, it is an adaptation which is very favorable to the persistence of these trematodes, since in infections in small snails it prevents the overcrowding of the digestive glands with secondary germinal sacs which would interfere with their development, and badly injure or kill the snail hosts.

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A *CUTEREBRA* LARVA (DIPTERA) FROM THE EPIDURAL SPACE OF A CAT

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Embalmed and injected cats are dissected routinely in the laboratory of comparative anatomy, University College, New York University. In one of these cats, purchased from Ward's Natural Science Establishment, Rochester, New York, and presumably from that region, a maggot was found in the epidural space on the left side of the brain and in the antero-dorsal portion of the cerebral fossa of the skull. It was identified by Curtis W. Sabrosky of the Insect Identification and Parasite Introduction ERB, U. S. National Museum, as a first-instar larva of a species of *Cuterebra*. Grateful acknowledgment is made to Mr. Sabrosky.

Maurice C. Hall (1921) reported on *Cuterebra* larvae from cats and gave a list of those recorded from other hosts. He described two larvae received on the same day by the Zoological Division of the U. S. Bureau of Animal Industry. One came from the nostrils of a cat in Nashville, Tennessee; it was apparently in the final stage, black and densely covered with spines, and measured 1.35 cm. long in a contracted condition. The other, from the neck of a cat in Washington, D. C., was in an earlier stage, white with small black spines along the anterior and posterior margins of the segments, and measured 6.4 mm. long in a contracted condition. Other sites of infection in cats, listed by Hall, were on the back, the belly, beside the eye, and under the jaw.

According to Matheson (1950), members of the genus *Cuterebra* are known only from North America. Their larvae are parasitic in rabbits and rodents and occur in tumors and warbles under the skin or in the nasal and pharyngeal cavities. They occur as accidental parasites in other mammals and Matheson cited two cases in man. He provided a key to the third-stage larvae of the more common flies that cause myiasis but stated, "Keys to the first-and second stage larvae are omitted because they are not of sufficient accuracy to warrant their use. Such larvae should be submitted to specialists."

The present account is apparently the first to report invasion of the cranial cavity by *Cuterebra* larvae, and although such infection is certainly rare, it may be more common than currently realized since the brains of cats, rabbits and rodents are seldom subjected to parasitological examination. The larva here reported had been preserved by the enbalming fluid and almost certainly was alive when the cat was killed. It measured 9 mm. in length, was curved ventrally, somewhat flattened laterally, but otherwise in good condition. Placed in a 0.25% solution of trisodium-phosphate, it swelled to normal appearance. Later it was stained, dehydrated and mounted in damar. The specimen is deposited in the collection of the American Museum of Natural History, New York. A camera lucida drawing (Fig. 1) presents anatomical details. It resembles somewhat the figure of a first stage larva of *Cuterebra americana* as portrayed by Ferris (1920). That

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species, *Musca americana* Fabricius, was named type of a new genus, *Atrypoderma,* by Townsend (1919). Other previously published figures of first stage cuterebrid larvae include: *Cuterebra* sp. from man by James (1948), *Cuterebra lepivora* from the western rabbit, *Sylvilagus audubonii sanctidiegi* by Ryckman and Lindt (1954), and *Cuterebra emasculator* from the eastern striped chipmunk, *Tamias striatus* Richardson by Bennett (1955).

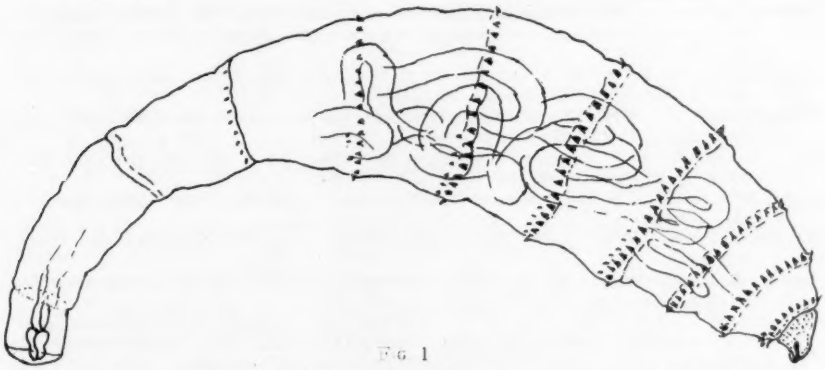

FIG. 1

There was no anatomical evidence to indicate the path by which the larva entered the brain case, but the habits of the adult flies and their larvae suggest possible routes. Parker and Wells (1919) reported that a female *Cuterebra tenebrosa* deposited 186 eggs, attached to the wall of a pasteboard box in which she was confined. Larvae removed from the eggs were very active and when placed in the mouth of a prairie dog, *Cynomys ludovicianus,* disappeared almost immediately. Some of them were recovered later. Eggs contained active larvae after six months in the laboratory. According to Ferris (1920), a female of *Cuterebra americana* deposited 100–150 eggs in 24 hours. Dalmat (1943) discussed the habits of adult flies of the genus *Cuterebra,* oviposition, and the methods by which their larvae gain entrance into their hosts. He stated that more than 1000 eggs had been taken from a female *Cuterebra horripilum,* a species whose larvae are reported to infect cats. The large number of eggs laid by these flies indicated that oviposition does not occur on the hosts, but among the nests, burrows and runways of these animals. Other studies on the life-cycles of cuterebrid species confirm this opinion. Beamer, Penner and Hibbard (1943) studied the developmental cycle of *Cuterebra beameri* from the pack rat, *Neotoma floridana osagenesis* Blair, in Kansas. They found eggs of the insect attached to objects near the entrances to the nests of the pack rats and reported two generations of the fly each year. Ryckman and Lindt (1954) described the stages in the life-cycle of *Cuterebra lepivora.* They were unable to infect domestic rabbits by placing larvae on the skin, but infection resulted from feeding larvae on lettuce. Bennett (1955) gave a detailed account of the genus *Cuterebra* and the life-history of *Cuterebra emasculator.* According to him the species of *Cuterebra* are almost specific for particular hosts. In the present instance, specific determination of the larva has not been made. It may have attached to or emerged on the mucous membrane of the nose of the cat, migrated to the ethmoid labyrinths of the nasal cavity, and passed through a perforation in the cribriform plate of the ethmoid bone; or it may have been licked from the cat's fur, pene-

trated the tissues of the oral cavity, and entered the skull by one of the foramina. Once in the epidural space, the rich blood supply to the meninges provided nourishment and oxygen.

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RESEARCH NOTE

TRICHINELLA SPIRALIS FROM THE URINARY BLADDER OF A PIG

In the course of an examination of experimentally infected trichinous hogs for the occurrence of the infective larvae of *Trichinella spiralis* in organs and tissues containing no striated muscle, an adult, gravid female of *T. spiralis* was recovered from the lumen of the urinary bladder of a young boar. Although movement was not observed, the appearance of the worm was that of a living nematode, or of one that had only recently died. The specimen was of normal size and did not appear to differ morphologically from normal trichinae. Partially developed embryos were present in the posterior portion of the uterus.

The host animal had been given ground, infected pork, estimated to contain 26,000 infective larvae, 54 days prior to necropsy. At that time, the animal weighed 26 pounds. Although 47,500 larvae, or 792 per gram of muscle tissue, were recovered from the diaphragm of this pig by artificial digestion, no larval trichinae were found in any of the organs and tissues not containing striated muscle tissue.

The examination was carried out in a manner which precluded contamination from any of the tissues of the pig, or from outside sources. Since the urinary bladder with its urethra, which had been tightly ligated close to the bladder wall, was the first organ to be removed from the freshly-killed animal, there appeared to be no opportunity for extraneous introduction of the worm.

So far as the writer is aware this is the first record of an adult *T. spiralis* occurring in the contents of the urinary bladder. The finding extends and supports the observation of McCoy (1936, J. Parasit. **22:** 54–59).—CHARLES H. HILL, *Animal Disease and Parasite Research Branch, ARS, Beltsville, Maryland.*

THE LIFE CYCLE OF *CRASSIPHIALA BULBOGLOSSA* (TREMATODA: STRIGEIDA). DEVELOPMENT OF THE METACERCARIA AND CYST, AND EFFECT ON THE FISH HOSTS

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INTRODUCTION

The adult of *Crassiphiala bulboglossa* was described by Van Haitsma (1925) from the kingfisher, *Ceryle alcycon*. It can be differentiated from *Uvulifer ambloplitis* (Hughes, 1927), apparently its closest relative in North America, by the presence of a large bulbous holdfast occupying most of a cup-shaped forebody, and the absence of a ventral sucker. In *U. amblopiitis* the holdfast is small, the forebody is not cupped, and a ventral sucker is present. These species can be readily identified with the keys given by Dubois (1953).

The metacercaria of *C. bulboglossa* was described as *Neascus bulboglossa* by Hughes (1928) from the perch, *Perca flavescens*. Van Haitsma proved it to be the metacercaria of *C. bulboglossa* by feeding experiments on kingfishers. It differs from all North American "*Neascus*" species by the absence of a ventral sucker (Hoffman, 1955a).

The life cycle of *U. ambloplitis* was determined by Krull (1934) and Hunter and Hunter (1934). Krull infected sunfish with the cercaria known as *Cercaria bessiae*, Cort and Brooks, 1928, from *Helisoma trivolvis* (Say), and found that the worm developed into *U. ambloplitis* (= *Neascus ambloplitis*). Hunter and Hunter studied the development in 2 species of snails, *Helisoma trivolvis* and *H. campanulatum*, and the resulting cercariae developed into *U. ambloplitis* in young small- and large-mouthed black bass, *Micropterus dolomieu* and *Huro salmoides*, rock bass, *Ambloplitis rupestris*, common sunfish, *Lepomis gibbosus*, and banded sunfish, *Enneacanthus obsessus*. Hunter and Hamilton (1941) observed the development of the cyst of *U. ambloplitis* in rock bass, common sunfish, and small-mouthed black bass, and found that the cyst of parasite origin appears in about 7 days and the host cyst becomes melanated during the third week. The nature of the cyst of *C. bulboglossa* in the skin of *Perca flavescens* was reported by Hunter and Hunter (1942).

Since *C. bulboglossa* and *U. ambloplitis* are closely related and have the same final host and species of *Helisoma* as molluscan hosts, as herein reported, one might expect that their developmental stages would be easily confused. The miracidium, sporocyst, and cercaria are very similar in the two species. Fortunately, fish and bird infections with *C. bulboglossa* predominated over infections with *U. ambloplitis* in the English Coulee, Grand Forks, North Dakota, and it was possible to complete the life cycle experimentally. This was briefly reported in abstract by Hoffman (1955b).

(Unless otherwise stated, the measurements given below are in microns)

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EGG AND MIRACIDIUM

Eggs of *C. bulboglossa* were obtained from feces and worms from 2 kingfishers taken in July and August, 1954. Both birds were heavily infected, 1 with at least 1,100 worms, so that many eggs were obtained. One worm in saline overnight expelled 68 apparently normal eggs. Ten eggs averaged 80 by 52, as compared with 90–104 by 56–70 for *U. ambloplitis* (Hunter and Hunter, 1934).

Eggs incubated in dechlorinated tap water (1 cc of 2% sodium thiosulfate per gallon) in standard Petri dishes at room temperature (23–25° C) yielded many miracidia. Some eggs still in the uterus of decomposing worms developed normally. The first miracidia hatched in 8–9 days.

The miracidia (Fig. 1) are very similar to those of *U. ambloplitis,* as described by Hunter and Hunter (1934).

Four living miracidia, quieted with 0.4% chloretone, measured 94–118 by 23–30 depending upon their state of contraction or expansion. Details are as follows: cilia 10 long, those at anterior tip about 5. Two small lateral papillae 5 long, about 15 from anterior end of worm; often a globule exuding from tip of papilla; about 6 cilia on papilla, possibly tactile in function; these cilia do not beat in chloretone solution although other cilia do. "Penetration organ" elliptical, 20 by 12, its 2 ducts easily seen, much enlarged within the "organ," extend back to unicellular penetration glands which are more difficult to see. Pigmented eye spots, crescentic in dorsolateral view, 7–8 in greatest diameter. One pair of flame cells just posterior to eye spots, another near posterior end of worm. A globular body about 12 long by 16 wide slightly posterior to eye spots, cf. "brain" described by Hunter and Hunter (1934) in *U. ambloplitis.* Similar body near posterior end appears to contain 2 round, dense structures; may be the germ ball. Hunter and Hunter do not describe these round structures in germ ball of *U. ambloplitis.*

DEVELOPMENT IN THE SNAIL

Many embryonated eggs were placed in a gallon jar of dechlorinated tap water containing 35 laboratory-reared snails, *Helisoma anceps* Menke. The snails were fed commercial fox chow, calcium was supplied with blackboard chalk or pure calcium carbonate, and compressed air forced into the jar furnished aeration. Water temperatures ranged from 23–28° C, averaging 24° C.

At 30 days post infection the liver of 1 experimentally infected snail was a tangled mass of sporocysts, almost impossible to separate. One freed sporocyst measured 3 mm by 50–100 and contained germ balls and cercariae, some of which appeared fully developed. Cercariae appeared at 33 and 34 days post infection from 6 out of 27 exposed snails in individual containers, as compared to 42 days for *U. ambloplitis* (Hunter and Hunter, 1934). Many cercariae were shed by the 6 experimentally infected snails during the 7 days after they first appeared, but only 5 snails shed worms for 9 days, and only 1 of these yielded many; only 1 snail shed a few worms at 11 days. No cercariae were shed during 35 days of further observation. The liver of 1 snail autopsied at 17 days after cercariae first appeared was a mass of sporocysts but only 1 to 4 cercariae were in each sporocyst. The same was true of a snail examined at 26 days.

Of 200 *Helisoma trivolvis* collected from August 18 to November 18, 1954, from the English Coulee, 1 was infected with *C. bulboglossa* and yielded cercariae for 10 days; after 20 days' refrigeration it yielded cercariae for only 1 day upon returning to room temperature.

Of 200 *Helisoma anceps* collected October 17, 1954, from Turtle River, 21 began to yield cercariae 13 and 14 days after collection. They were refrigerated for 11 to 28 days but shed no more cercariae upon returning to room temperature.

Three more snails began to shed cercariae 25 days after collection and stopped after 5 days at room temperature. Four more began at 30 days after collection and shed worms for 2, 3, 6 and 16 days, respectively.

No accurate record was kept of the longevity of infected snails, but some died as soon as 15 days after cercariae first appeared and all were dead in 2½ months. Many uninfected snails survived similar conditions for longer lengths of time with less mortality. Although laboratory and natural conditions are not entirely comparable, it is likely that most infected snails die during the year, but that those infected late in the season probably survive the winter as the sporocysts develop slowly and then produce cercariae for a while the next spring.

THE CERCARIAE

The cercaria (Figs. 2, 3, 4) differs only slightly from that of *U. ambloplitis* (= *Cercaria bessiae*) as described by Cort and Brooks (1928) from *Helisoma lantum* from Michigan and redescribed by Miller (1936) from *H. trivolvis* from Louisiana. The cercariae used here were obtained from experimentally infected *H. anceps* and naturally infected *H. anceps* and *H. trivolvis*. All cercariae were proven to be *Crassiphiala bulboglossa* by recovering typical metacercariae from fish experimentally infected with the cercariae from the same snail. The writer believes that the metacercaria of this species is distinctive enough for species identification (see key in Hoffman, 1955a). The cercaria hangs in the water with the body downward and bent like a hook. Occasionally a rapid side to side motion of the tail drives the larva tail-first upwards in the water. Its behavior strikingly resembles that of the cercaria of *U. ambloplitis*. Maximum longevity was 25½ hours.

The following measurements and observations were made on living specimens with the aid of the intra-vital stains neutral red and nile blue sulfate, and sometimes with 0.4% chloretone as an anesthetic: Body length of 23 cercariae, relaxed (or nearly so) 106–230, average 152; width 28–54, average 41. Tail stem, length 190–360, average 250; width, 24–42, average 31. Furcae, length 190–360, average 250; width, 24–42, average 31. Furcae, length 180–320, average 226; width from lateral view 30, from ventral or dorsal view, about 14. Body usually a little wider than tail stem, usually show 7–8 muscular annulations when extended. Anterior fourth of body with minute spines just visible under oil immersion. One pair posteriolateral flagellets about 5 long, on body. Penetration organ oval, 23–30 by 18–28. Three pairs penetration glands in posterior half of body, ducts easily seen in region of penetration organ. Gut short, rhabdocoel, difficult to see. Ventral sucker absent. Eye spots not seen. Nine flame cells on each side, one just posterior to penetration organ, second just posterior to first, 4 at irregular intervals near penetration glands, seventh at anterior level of excretory bladder, 2 in basal half of tail stem. Bladder at posterior end of body, constricted into 2 parts: anterior portion about 8 long by 15 wide, receives 2 collecting tubules; posterior bulbous part about 6 diameter, joins caudal excretory tubule. Heavily nucleated body about 12 in diameter just anterior to bladder is assumed to be genital primordium. Tail stem has about 25 annulations, approximately 10 long, regularly spaced; 10–15 flagellets each side, 15–25 long, closer together near ends of tail stem than at middle. Small globules sometimes seen at ends of flagellets; flagellet appears shorter as globule becomes larger, as seen by Cable (1955) in other cercariae. Two lateral rows of about 40 cells with conspicuous nuclei and a central "core" of about 75 prominently nucleated cells in tail stem. Each furca with 8 longitudinal rows of minute spines about 1 long on each side giving a striated appearance; each furca contains 2 rows of about 25 prominently nucleated cells.

The cercariae used by Krull (1934), Hunter and Hunter (1934), and Hunter and Hamilton (1941) in obtaining metacercariae of *Uvulifer ambloplitis* were identified as *Cercaria bessiae* but were not described. The cercaria of *Crassiphiala bulboglossa* differs only slightly from *Cercaria bessiae* as described by Cort and Brooks (1928) and Miller (1936), in the following respects: (1) Tail stem

longer and narrower, (2) Furcae longer and wider, (3) Seven flame cells on each side of body instead of 6, (4) Unpigmented eye spots, if present, could not be seen, (5) One pair of posterio-lateral flagellets on the cercaria of *C. bulboglossa* but not on *C. bessiae*.

(The writer wishes to acknowledge with gratitude the use of a mimeographed key to the furcocercous cercariae (1953) kindly supplied by Dr. Asa C. Chandler.)

DEVELOPMENT OF THE METACERCARIA

The metacercaria of *Crassiphiala bulboglossa* has been recorded from 11 species of 6 families of fishes (Host record verified by Allen McIntosh, Zoology Div., Bur. Animal Ind., Beltsville, Maryland): CYPRINIDAE—*Ericymba buccata, Leucosomus corporalis, Notemigonus crysoleucas, Notropis cornutus frontalis, Semotilus a. atromaculatus;* CYPRINODONTIDAE—*Fundulus diaphanus;* ESOCIDAE—*Esox lucius;* ETHEOSTOMIDAE—*Boleosoma nigrum olmstedi;* PERCIDAE—*Perca flavescens, Stizostedion vitreum;* UMBRIDAE—*Umbra limi.* It is probable that many other species of fish are susceptible.

Black spot *Neascus*-free individuals of 4 species of fish, fathead, *Pimephales p. promelas,* killifish, *Fundulus diaphanus menona,* brook stickleback, *Eucalia inconstans* and common black sucker, *Catostomus c. comersonnii* were exposed to variable numbers of cercariae in a small amount of dechlorinated tap water in gallon jars at 21 to 25° C. Of the 39 killifish exposed, 38 became infected; all of 22 fatheads became infected; 7 of the 8 sticklebacks became infected and the 1 sucker did not become infected, even though it was exposed to cercariae on 2 different occasions. During the development of the metacercariae in the fish, the temperature ranged from 21–25° C, averaging about 23° C. In 1 lot kept at 25° C continuosly, metacercariae became fully developed about 2 days sooner than in other lots.

The larvae readily penetrated the fatheads, killifish, and sticklebacks and could be recovered from the skin in large numbers after 4 days. The developing metacercariae were examined at nearly daily intervals but for convenience the observations will here be given for only those ages which show some morphological difference over preceding ones. The stages are given as they occur at 21–24° C, averaging 23° C. At 25–26° C all stages occur a day or so earlier.

Four days (Fig. 5)—Larvae found free in skin. No evidence of cysts either of host or parasite origin. Larvae not found earlier probably because of small size and numbers. Alive and relaxed, measured *ca.* 148 by 54; very active at that age, largest individual 180 by 50 extended, 120 by 110 contracted. Larva often ameboid, nearly round, occasionally moves to side by forming projection grossly like a pseudopod. Usually anterior part, containing penetration organ, extends slightly forward (Fig. 6). Phillips (1955) states that the metacercaria of *Troglotrema salmincola* was once described as an ameba. Fixed and stained specimens, like fresh ones, show only cercarial penetration organ; body stains very heavily.

Six days—Cyst of host origin just discernible in skin of fresh fish at 10× magnification. Metacercaria slightly larger, about 188 by 74, relaxed; otherwise no morphological change detected.

Seven days—Accumulation of black pigment around cysts evident in fatheads and stickleback, not in killifish. Pigmentation is first reported at 3–7 weeks for *U. amblopitis* (Krull, 1934; Hunter and Hunter, 1934). Congested or enlarged blood vessels noticeable about cysts especially in heavy infections. Bases of fins, opercula, ventral surface of head hyperemic in heavily infected fish and this condition usually persists almost until the metacercaria is fully developed. Host cyst 9–16 thick in histological cross section, composed of unflattened cells 7–9 in diameter. No connective tissue fibers visible.

Fourteen days—(Fig. 7)—Cysts heavily pigmented in fathead and stickleback, no pigment in killifish with one exception. Host cysts, exclusive of outer pigmented layer, 300–370 by 280–300, *ca.* 42 thick Strigeid characteristics first appear in metacercaria, hindbody a small conical posterior projection, forebody suggests leaf-like form of some strigeids; holdfast a bulbous projection *ca.* 66 in diameter. Relaxed worm *ca.* 245 by 150, contracted, 185 by 165. Oral sucker distinct, *ca.* 21–28 diameter. Pharynx subspherical, 18 wide. Reserve excretory system and cyst of parasite origin not evident. In stickleback, metacercariae do not develop this far; never develop strigeid constriction or cyst of parasite origin, but do develop rudimentary holdfast; remain alive 24–81 days, finally die and are resorbed; melanated host cyst remains intact long after death of parasite, at least 81 days post infection.

Sixteen days (Figs. 8, 9)—Metacercaria has typical *Neascus* shape, *ca.* 322 by 165, definite conical hindbody *ca.* 94 by 71. Ceca not evident in live specimens; present as very thin tubes in stained specimens. Body nuclei still very large, internal structure of worm difficult to see. Entire worm appears "vacuolated." Large bodies, 10 by 25 in holdfast and entire forebody, varying in shape from oval and elongate to nearly round but irregular; rhizoid extensions occur at ends of some large elongate ones. The writer believes these are cystogenous glands because they disappear after cyst of parasite origin is formed. In histological section there are numerous vacuoles which are probably the same. Holdfast still bulbous, 90 in diameter. Reserve excretory system not evident.

In section the host cyst appears as 2 layers, an inner cellular one about 6–10 thick composed of cuboidal cells in irregular rows 1 to 3 cells deep, and an outer connective tissue layer 4–36 thick, composed of many flattened cells and fibers. Melanophores, when present, lie in a loose layer in outer margin of cyst.

Seventeen and eighteen days—Metacercaria attains maximum size, 310–400 by 180–215. Hindbody 106–120 by 54–56. Bulbous holdfast about 94 long by 110 wide in ventral view. Forebody wide, not conspicuously "cupped" yet. Whole worm, including holdfast, looks swollen and thickened during at least part of period; large cells or vacuoles present in holdfast of live specimen.

Nineteen days (Fig. 10)—Worm somewhat flattened, including holdfast, forebody somewhat "cupped." Main branches of reserve excretory system as described for mature metacercaria by Hughes (1928) readily visible, contain rounded bodies, the largest 10 in diameter. Before formation of reserve excretory system, a quantity of granular material in forebody seems to deliniate future excretory tubules; it is absent after tubules are formed. Excretory bladder in hindbody has 2 main arms which join near excretory pore; each has a slight constriction about midway. Inner part of host cyst can be easily peeled from outer pigmented layer. Parasite lacks parasite cyst, can easily be forced out of host cyst.

Twenty days (Fig. 11)—Parasite cyst forming around some individuals; time of formation depends on temperature and number of metacercariae in fish, being delayed 4 or 5 days in a heavily parasitized fish. Newly secreted parasite cyst material gelatinous looking, very pliable, 9–23 thick. Metacercaria rounded, folded in typical *Neascus* manner as described by Hughes (1928). This time interval contrasts strikingly with 6–8 days for parasite cyst formation in *U. ambloplitis* (Hunter and Hamilton, 1941), but is comparable to 20–21 days in *Posthodiplostomum minimum centrarchi* (Hoffman, 1950). Host cyst no longer shows cuboidal inner cells, but entire cyst, 23–26 thick, composed of compressed connective tissue. Looser melanophore layer when present is on outer edge of cyst (see description given by Hunter and Hunter, 1942).

Twenty-one to twenty-two days (Figs. 12, 13, 14)—At *ca.* 23°C all cysts of parasite origin formed, congealed, and contracted to normal mature thickness of 1–2. Metacercaria appears as described by Hughes (1928), but ellipsoidal parasite cysts in writer's material measured 189–260 in length, 147–190 in lateral width (average 241 by 164), *ca.* 108 in dorsoventral depth; Hughes' specimens averaged 200 by 160. Host cyst, excluding outer pigmented layer, diameter totals *ca.* 600. Most cysts were just beneath epithelium of fish, either above or below a scale, but several were found on gill arches and under epithelium of gullet in heavily infected fish. Many nuclei appear in mature cysts, but fewer than in younger cysts. Dense inner layer 22–49 thick, loose outer layer 0–50 thick in oldest specimens studied, 60 days.

Mature metacercariae can be removed from cysts by digestion with either pepsin (Hoffman, 1955a) or trypsin (Hughes, 1928).

Fischthal (1949) found that black grubs (*Neascus spp.*) and yellow grubs (*Clinostomum marginatum*) survived "wintering over" in fish. The author has attempted to keep *Pimiphales p. promelas* infected with *Crassiphiala bulboglossa* and *U. ambloplitis* in the laboratory for long periods to study longevity of *C. bulbo-*

glossa. The fish usually do not survive much over a year, but in one which survived 37 months, all of the worms examined of both species were still alive. Hoffman (1950) found that another strigeid, *Posthodiplostomum minimum centrarchi* survived at least 11 months in naturally infected fish in laboratory aquaria.

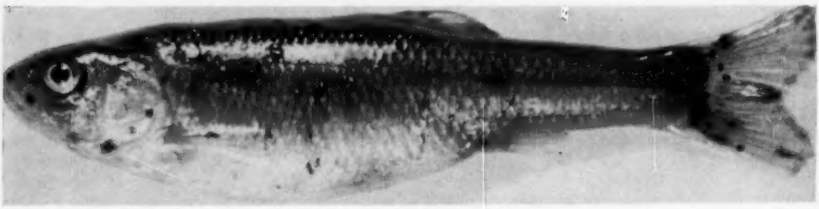

FIG. 14. Fathead, *Pimephales p. promelas*, with fully developed cysts of *Crassiphiala bulboglossa*.

HOST DIFFERENCES

It has already been stated that the stickleback becomes infected but the parasite does not attain full development and probably does not become infective in that fish. It lives up to 81 days in this apparently abnormal host and eventually dies; the writer is not aware of experimental demonstration of this phenomenon in other fish trematodes, and it may be unusual.

On several occasions killifish taken from the same experimental jar as fatheads contained metacercariae which were one to several days younger in appearance than those of the fatheads. One heavily infected fathead 2½ inches long contained about 600 cysts in which the parasites did not secrete the parasite cyst until 5 days after those of similar but less heavily infected fish.

The pigmentation of the cyst in the fathead was similar to that occuring in other fish, except that there were many cysts with accumulated xanthophores instead of melanophores about them. The majority of these yellow cysts occurred in the less heavily pigmented areas of the fish, i. e., the ventral and ventro-lateral areas. Similar yellow pigmentation has been reported by Chapman and Hunter (1954) for cysts of *Cryptocotyle lingua* in the cunner, *Tautoglabrus adspersus*.

Pigmented cysts were observed in but 1 of the 38 killifish studied. The accumulation of pigment cells was not great but was definite around 2 cysts on the caudal fin. Normally, the pigment cells are close to the fin rays in the killifish but on this occasion some of them appeared to have migrated down the ray to the cyst and a row of pigment cells could be seen along the ray on either side of the cyst. The mechanisms of the pigmentation of parasite cysts is not known. Hunter and Hamilton, (1941), theorized that the cells of the fish contain the enzyme necessary for pigment production and that the parasite, in some way, provides chromogen or vice versa. Chapman and Hunter (1954) applied the dopa test to the developing cysts at 7–33 days (the time during which pigmentation occurs) and elucidated no evidence of dopa oxidase. They also saw no evidence of melanophore migration into the area of the cyst. Therefore, the mechanism still remains to be determined.

EFFECT OF THE PARASITE ON THE FISH

Krull (1934) reported that the cercariae of *Uvulifer ambloplitis* produce a spectacular nervous response in fish, and heavily infected fish die in 2–4 days. Hunter

and Hunter (1938) observed that heavy infections caused significant weight loss. The writer exposed 2 adult fatheads to as large a concentration of cercariae as could be obtained. At no time, however, did these fish demonstrate severe nervous symptoms. At 7 days the fish were "peppered" with developing black-spot cysts, and there was extensive congestion of blood vessels at the bases of the fins and ventral and posterior portions of the opercula. Histological examination demonstrated severe congestion but no hemorrhage. Vascular enlargement also occurred around the cysts themselves but was much more evident in the locations just mentioned. One fish which seemed most affected died at 10 days post infection and was literally covered with developing cysts. The other one, although in distress, was more lethargic than nervous. The scales were raised over the entire body giving the fish a very rough appearance. At 18 days it was not feeding, but at 21 days it was taking food and seemed livelier. A part of a fin containing cysts was removed each day to study the development of the metacercariae. At 25 days, 4–6 days later than in less heavily infected fish, the parasite cyst was formed and the fish was autopsied. Approximately 600 cysts were present.

On another occasion each of 6 killifish about 2½ inches long were exposed to 400, 800, 1600, and 2600 cercariae, respectively. The fish exposed to 400 cercariae developed a small degree of hyperemia at the bases of the fins, but appeared normal in other respects and was preserved at 27 days when the cysts of parasite origin were being formed. The fish exposed to 800 cercariae demonstrated much vascular congestion by 15 days, was not feeding at 22 days and went into a sort of frenzy when disturbed. The fish exposed to 1600 cercariae had developed much vascular congestion at 15 days, demonstrated the same frenzy as the preceding fish, and died at 19 days. The fish exposed to 2600 cercariae showed much congestion as early as 9 days, and at 15 days there were spotty hemorrhages over the entire body. At 16 days the fish died and was estimated to have 400 cysts. They were so numerous that in some instances 2 or 3 parasites were enclosed in a single host cyst.

These observations on the effect of the parasite on the fish are rather cursory, but they do show that the type of damage done must be considerably different from that reported by Krull (1934) for *U. ambloplitis*. Under natural conditions it is doubtful that a fish would be exposed to as concentrated a dosage of cercariae as was used experimentally, and except under very unusual circumstances, this parasite probably would not contribute to the mortality of the fish.

IMMUNITY

Krull (1934) reported that older sunfish previously infected with a few *Uvulifer ambloplitis* were refractory to further infection. Ferguson (1943) and Hoffman (1950) were able to reinfect previously infected cyprinid fish with *Posthodiplostomum minimum*. The writer has been able to reinfect previously infected fish with *Diplostomulm baeri eucaliae*. In the present study, 4 fatheads, previously infected with *Crassiphiala bulboglossa*, and 6 uninfected fatheads as controls, were exposed to the cercariae of *C. bulboglossa;* the 6 control fish became infected and the other 4 were autopsied at 16 days or less and examined for superimposed *C. bulboglossa* metacercariae; the superimposed metacercariae could easily be differentiated from those of previous infection because they lacked the cysts of parasite origin which had already been formed in the older natural infec-

tions. In all cases the fish did become successfully reinfected with numerous parasites and no evidence of immunity was observed.

SUMMARY

1. The life-cycle of *Crassiphiala bulboglossa* was completed by experimentally infecting the snail, *Helisoma anceps,* with miracidia hatched from ova obtained from adult *C. bulboglossa* from the kingfisher, *Ceryle alcyon,* and subsequently infecting fish, *Pimephales p. promelas* and *Fundulus diaphanus menona,* with the resulting cercariae.

2. The miracidium and cercaria are described.

3. The development of the metacercaria and its cysts are described.

4. Large numbers of cercariae killed fish but not until 10–15 days post infection. No evidence of immunity was observed.

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PLATE I

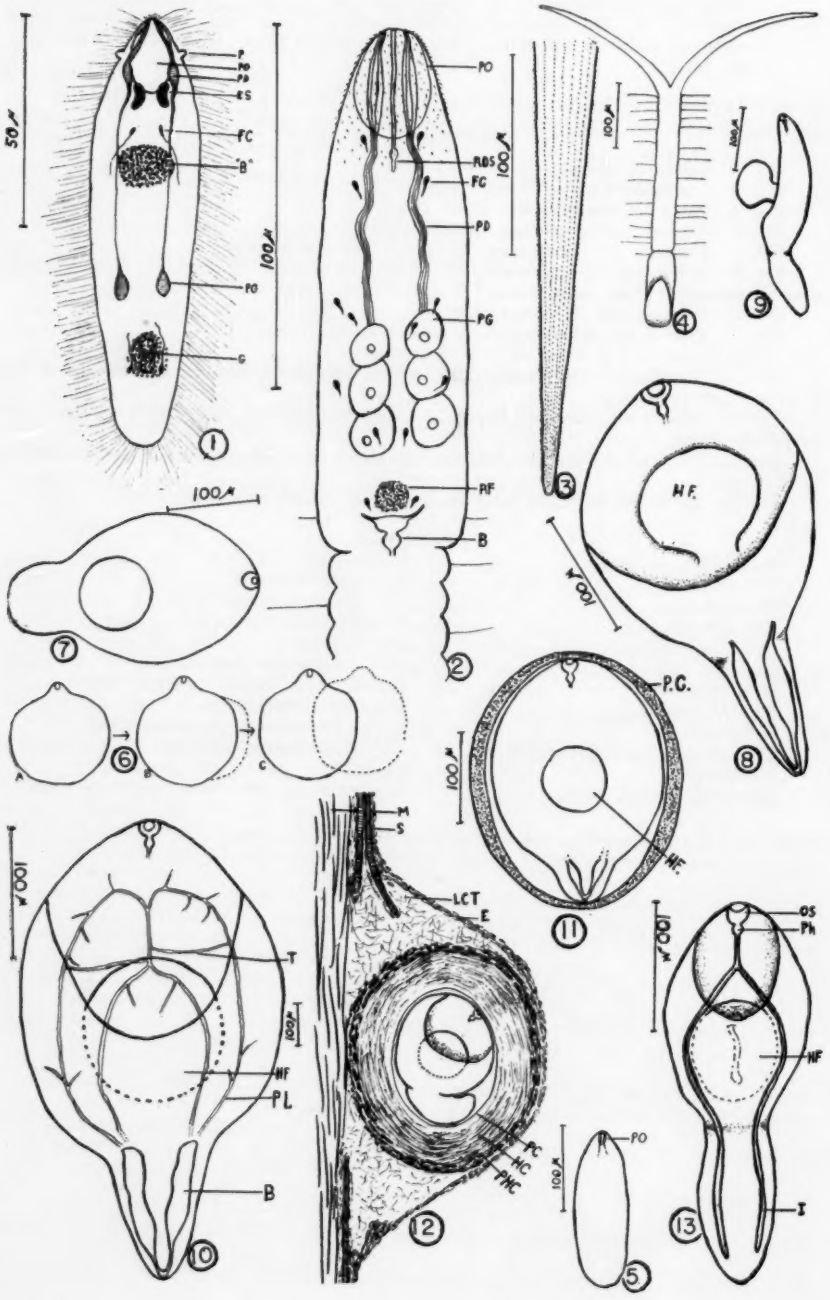
Explanation of Figures

FIG. 1. Composite drawing of the miracidium.
FIG. 2. Composite drawing of the cercaria.
FIG. 3. Furca as seen from the lateral view.
FIG. 4. Normal resting position of the cercaria.
FIG. 5. Four-day old metacercaria in normal extended position.
FIG. 6. A. Four-day old metacercaria in contracted position. B and C. Sketches showing ameboid movement of the metacercaria.
FIG. 7. Fourteen-day old metacercaria.
FIG. 8. Sixteen-day old metacercaria freed from host cyst.
FIG. 9. Same as Fig. 8, but from lateral view.
FIG. 10. Nineteen-day old metacercaria showing the newly formed main tubules of the reserve excretory system.
FIG. 11. Twenty-day old metacercaria showing the relatively thick newly secreted cyst of parasite origin.
FIG. 12. Freehand drawing showing the appearance of a fully developed cyst as seen in cross section.
FIG. 13. Mature metacercaria freed from its cyst; excretory system omitted.

Abbreviations

B—Bladder, excretory
"B"—Brain
E—Epithelium
ES—Eye-spot
FC—Flame cell
G—Germ ball
HC—Host cyst
HF—Hold-fast
I—Intestine
LCT—Loose connective tissue
M—Muscle
OS—Oral Sucker
P—Papilla
PC—Parasite cyst
PD—Penetration duct
PG—Penetration gland
Ph—Pharynx
PHC—Pigmented host cyst
PL—Primary lateral excretory vessel
PO—Penetration organ
RDS—Rudimentary digestive system
RF—Reproductive fundament
S—Scale
T—Transverse commisural excretory vessel

PLATE I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STUDIES ON EGYPTIAN TREMATODES WITH SPECIAL REFERENCE TO THE HETEROPHYIDS OF MAMMALS. I. ADULT FLUKES, WITH DESCRIPTIONS OF *PHAGICOLA LONGICOLLIS* N. SP., *CYNODIPLOSTOMUM NAMRUI* N. SP., AND A *STEPHANOPRORA* FROM CATS*

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The present report is based upon a study of a part of a rather extensive collection of helminths from vertebrates and is concerned primarily with heterophyids and accompanying trematodes obtained from cats, several other mammals and a few birds taken in Lower Egypt. The second paper in this series, in preparation, will give the development of the redial and cercarial stages of *Heterophyes aequalis* in the brackish water snail, *Pirenella conica*.

The authors are indebted to Lt. W. H. Wells, MSC, USN and George M. Malakatis, HM1, USN, of U. S. Naval Medical Research Unit No. 3, Cairo, for assistance in collection of materials.

MATERIALS AND METHODS

Parasites for this study were obtained from 48 domestic cats, 3 dogs, 1 fox (*Vulpes vulpes*), 2 shrews (*Crocidura olivieri*), 1 rat (*Rattus rattus*) and a few birds (5 kites, *Milvus migrans;* 1 osprey, *Pandion* sp.; and 1 domestic duck). All cats, dogs and the fox were taken alive, transported to the laboratory and examined within 3 days. Shrews and the rat were caught in live traps whereas all birds were shot. Some of the latter were examined in the field at the sites of collection while others were held 4 to 8 hours until examined in the laboratory. Many of the smaller trematodes were recovered from the settlings after repeated washing and shaking of parts of intestine in physiological saline. Specimens were killed in hot water and then transferred immediately to FAA (formalin-acetic acid-alcohol). After 8 to 24 hours in fixative the trematodes were transferred to 70 per cent alcohol plus 1 per cent glycerine for storage.

DISCUSSION AND DESCRIPTION OF PARASITES

A total of 10 species of heterophyids was obtained, 1 of which is new. There were 2 species representing the Echinostomatidae, including 1 (*Stephanoprora denticulatoides*) not previously reported from cats or from Egypt. One new species of trematode is described for the Diplostomatidae and for the Cyathocotylidae. All of these were present in cats. Table I shows the incidence of parasites and lists the geographic localities from which hosts were obtained. The cats from Cairo were from different parts of the city but there was no correlation between place of collection and parasites harbored. It is probable that the majority

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* The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

TABLE I. Hosts, geographic locality and incidence of heterophyids and accompanying trematodes

LOCALITY	NUMBER EXAMINED	HETEROPHYES HETEROPHYES	HETEROPHYES AEQUALIS	HAPLORCHIS PUMILIO	HAPLORCHIS YOKOGAWAI	HAPLORCHIS TAICHUI	STELLANTCHASMUS FALCATUS	PHAGICOLA LONGICOLLIS n. sp.	PHAGICOLA ASCOLONGA	PYGIDIOPSIS GENATA	STETTODORA HAWAIENSIS	ECHINOCHASMUS LILIPUTANUS	STEPHANOPRORA DENTICULATOIDES	MESOSTEPHANUS APPENDICULATUS	CYNODIPLOSTOMUM KAMRUI n. sp.
Cats															
Medit. Coast	16	16	7	15	3	0	16	11	8	8	0	3	3	1	1
Cairo	19	18	3	16	8	0	8	10	9	14	1	6	0	10	5
Giza	9	2	1	8	8	1	2	1	2	2	0	1	0	6	1
Salum	3	2	1	0	0	0	0	0	0	0	0	1	0	0	0
Faiyum	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Total:	48	39	13	40	16	1	33	13	17	24	1	11	3	17	7
Dogs															
Cairo	3	3	1	2	2	1	1	0	0	1	0	2	0	3	2
Fox															
Giza	1	1	1	1	0	0	0	0	0	1	0	1	0	1	0
Shrews															
Giza	2	0	0	2	0	0	1	0	0	1	0	0	0	0	0
Rat															
Cairo	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Kites															
Faiyum	5	0	1	1	1	0	0	0	0	3	0	0	0	0	0
Osprey															
Faiyum	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Duck															
Faiyum	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0

had fed on market fish imported from the coasts as well as fresh water fish from the Nile. Kittens and pups developed pure infections with *Heterophyes aequalis* after they were fed metacercariae from laboratory-infected *Gambusia affinis* which had been exposed to selected heterophyid cercariae from snails (*Pirenella conica*), collected at El Burg on the Mediterranean coast. Wells (unpublished data) working in the same laboratory, also obtained *H. aequalis* from experimental feedings to domestic rabbits and white rats. Furthermore, he found fish (*Tilapia nilotica* and *Solea vulgaris*) naturally infected with the metacercariae of *H. aequalis* in the inland brackish Lake Qârûn. Adult flukes were obtained from laboratory feedings.

The cats from Giza Province were in contrast to those from other localities in that only 2 of 9 harbored *Heterophyes heterophyes* and none *H. aequalis*. *Haplorchis pumilio, H. yokogawai* and *Mesostephanus appendiculatus* were the commonest flukes in these cats. Distibution of these species suggests fresh water intermediate hosts.

Although the majority of the species found by us in cats and dogs are the same as reported in other surveys of cats and/or dogs in Egypt, there were a few striking exceptions. We found two species of *Phagicola* (Faust, 1920) Price 1932; one *P. ascolonga*, was previously reported by Gohar (1934a; 1935) and Abdel Azim (1938) in Egyptian dogs and kites (*Milvus*) but the other is a new species, described below. On the other hand we failed, as did Abdel Azim (1938), to find *P. minuta*, which Looss (1899) described from cats and dogs in both Cairo and

Alexandria and which Gohar also listed from Egyptian hosts. We failed also to find *P. longa* and *P. italica,* both of which Abdel Azim found more frequently than *P. ascolonga* in dogs in Lower Egypt. Also peculiar was the absence of *Prohemistomum vivax,* which Abdel Azim found commonly in dogs in Cairo and Upper Egypt, but the frequent occurrence of the fairly closely related *Mesostephanus appendiculatus,* not previously reported from Egypt. We also found what we believe to be a new species of *Cynodiplostomum.* We also failed to find *Echinochasmus perfoliatus* which Abdel Azim reported as common in dogs in Lower and Upper Egypt, although we found *E. liliputanus* Looss, 1896 previously reported in Egypt only from accipitrine birds (*Milvus migrans* and *Pernis apivorus*). The finding of *Stephanoprora denticulatoides,* previously known only from dogs in Crimea, was unexpected inasmuch as Abdel Azim (1938) failed to find it in 320 dogs from Lower Egypt, 100 of them from Alexandria.

Heterophyes

On the basis of our material from Egypt plus some from the Yemen, we feel compelled to reduce the Middle East species of this genus to only two, *H. heterophyes* and *H. aequalis,* reducing *H. dispar* which Witenberg (1929) retained as a separate species, to synonymy with *H. aequalis.* These two species are easily distinguishable by the small number (15 to 35) of small, well spaced rodlets on the gonotyl in *H. aequalis* and the numerous (70 to 85) larger, closely set and comb-like rodlets in *H. heterophyes. H. aequalis* and *H. dispar* were differentiated by Looss (1902) and by Witenberg on the basis of (1) the relatively larger size of the ventral sucker as compared with the oral sucker in *H. dispar* and (2) the inflated ceca, reaching to the level of the testes, but not beyond, in *H. aequalis,* whereas in *H. dispar* the ceca are narrow and bend inward behind the testes. In addition Witenberg described *H. aequalis* as having 15 to 25 rodlets on the gonotyl, and *dispar* as having 25 to 35.

In our Egyptian material we have found specimens with relatively large ventral suckers and long ceca with only 17 to 20 rodlets. In both natural and experimental infections in dogs and cats we have found specimens with the oral sucker ranging in transverse diameter from 45 to 68 microns with the length somewhat less, and the ventral sucker from 53 to 125 microns in diameter, although it seldom exceeded 100 microns. There was no correlation between the relative size of the ventral sucker and the extent of the ceca to or behind the testes, nor either of these characters with the inflation of the ceca. Our belief is that when the ceca appear broad and inflated they tend to shorten in length and fail to bend around behind the testes. It is also evident that the testes vary in their distance from the posterior end of the body and may move with contractions of the body. In some specimens from the Yemen we found the oral sucker to range from 52 to 73 microns in diameter and the ventral sucker from 75 to 150 microns. In these the ceca were variable in posterior extent. In some specimens neither, and in others, one or both extended behind the corresponding testis. In 1 of 5 kites (*Milvus migrans*) we found specimens of *H. aequalis* which were very small, 0.37 to 0.45 mm long, with the oral sucker only slightly smaller than the ventral sucker (35 to 50 microns, average 43; and 37 to 50 microns, average 46, respectively).

In the Egyptian collections *H. heterophyes* invariably accompanied *H. aequalis* in naturally infected mammals, and in much larger numbers, although we found

pure infections with *H. aequalis* in some laboratory-reared hosts (cats, rats, rabbits) fed on metacercariae teased from musculature of *Gambusia* infected with cercariae from *Pirenella* (see part II of this series). Our findings as well as those of Abdel Azim (1938) suggest dependence on marine or brackish water hosts.

H. heterophyes was the second most common fluke found by us in Egyptian cats and dogs. It was also found in cats from the Yemen (Hodeida and Sana') and was obtained from a man from Baltim on the Mediterranean coast . A single specimen was taken from a kite. Macy (1953) reported *H. heterophyes* from a bat which the senior author (REK) examined in the Yemen.

Looss (1902) differentiated a subspecies, *H. heterophyes sentus,* common in cats but rare in dogs, on the basis of the smaller body size and relatively smaller ventral sucker. Witenberg (1929) did not recognize Looss's subspecies but called attention to the fact that *H. heterophyes,* in natural and experimental infections, tends to be larger in dogs than in cats; that in dogs the body is pear-shaped, in cats tongue-shaped; and that in cats the ventral sucker tends to be relatively smaller.

All of these differences are well marked in the majority of our specimens from Egypt. Specimens from man and fox resemble those from dogs. In cats our specimens range in size from 0.5 to 1.4 (rarely 1.6) mm with a width of 0.25 to 0.55 mm. Frequently the length is from 4 to 5 times the width and only rarely as little as 2½ to 3 times. In the other hosts the length ranges from 1 to 1.6 mm by 0.34 mm to 0.6mm, with the length from 2½ to 3½ times the width. In specimens from dog, fox and man the ventral sucker is almost always situated in the middle of the body or slightly anterior, whereas in the majority of specimens from cats it is posterior to the middle of the body. In extreme cases the ratio of anterior and posterior lengths is 5 : 3. In cats the ventral sucker ranges from 60 to 180 microns in diameter, except in two specimens—one from Egypt and one from the Yemen—in which it measures 230 and 195 microns, respectively. In specimens from the other hosts, the diameter of ventral sucker ranges from 185 to 310 microns. There is a marked difference in the size of the ventral sucker in individual cats, e.g., 60 to 87, 83 to 115, 105 to 180 and 140 to 150 microns.

Haplorchis

Haplorchis was the most comon fluke found by us in Egyptian cats and dogs. There has been considerable confusion concerning the species in this genus of extremely small flukes in which the hindbody is often so filled with eggs that few details of structure can be seen. As Chen (1936) pointed out, the only constant specific difference practical enough for diagnosis is the structure and armature of the gonotyl. *Haplorchis pumilio,* however may be recognized by the relatively short ceca. These flukes are remarkably indiscriminate with respect to the metacercarial hosts which include fresh, brackish water and marine fishes. Also, the definitive hosts have been found in natural infections in a great variety of fish-eating birds and mammals. Experimental infections suggest that maturity may be reached in almost any warm-blooded animal ingesting the metacercariae. It is not surprising that several species have wide geographical distribution, extending from Tunis, Egypt and Palestine to Formosa and the Philippines. Reviews of this genus and the forms related to it in the subfamily Haplorchiinae have been given by Chen (1936, 1949).

Three species of *Haplorchis, H. pumilio, H. yokogawai* and *H. taichui,* were taken from Egyptian mammals and birds (Table I). *H. pumilio* was the most common and found in greatest variety of hosts. This species was first described by Looss (1896) from a pelican (*Pelecanus onocrotalus*) and a kite (*Milvus migrans*) in Egypt. It was subsequently described by Faust and Nishigori (1926) under the name *Monorchotrema taihokui* from naturally or experimentally infected birds and mammals in Formosa. Africa and Garcia (1935) reported it from man and dogs in the Philippines but Africa (1938) stated that the species involved is really *H. yokogawai* (see below). Witenberg (1929) reported *H. pumilio* as a rare parasite of dogs and cats in Palestine and found it once in a gull (*Larus* sp.). Gohar (1935) reported three species of *Haplorchis* from Egyptian kites under the names *H. pumilio, H. taichu,* and *H. milvi.* His description of the gonotyl of *H. pumilio,* especially the length of the ceca and the size of the eggs, makes it evident that this form was actually *H. yokogawai.* On the other hand, his *H. milvi,* described as a new species (1934b) is probably *H. pumilio.* Gohar described the genital sucker as having a crown of 60 to 70 spines, instead of the 36 to 40 characteristic of *H. pumilio.* Some of the peculiarly shaped spines, or preferably hooks, of *H. pumilio* which are well figured by Chen (1936), sometimes appear as two spines instead of one, especially when the sucker is viewed in certain positions. The size of the eggs of *H. milvi* also conforms to that of the eggs of *H. pumilio.* The extent of the ceca was not determined.

In *H. pumilio* the broader posterior portion of the body is essentially round in cross section, so that mounted specimens frequently lie in a lateral or semi-lateral position. The gonotyl is shaped like a cone ending interiorly in a bluntly rounded tip directed medially and posteriorly from the body surface. The broad end of the cone, projecting as a blunt triangle in laterally mounted specimens (Plate I, Fig. 1) is armed with an almost complete crown of spines or hooks as described by Chen (1936). In specimens mounted in optimal position, two groups of finer spines, presumably surrounding the openings of sex ducts, may be seen inside the gonotyl, as described by Looss (1896). This species is further characterized by the shortness of the ceca which have a large lumen and end near the level of anterior border of the testis, whereas in other species of this genus they extend to the posterior end of testis. Eggs of *H. pumilio* are somewhat larger than those of *H. yokogawai* and *H. taichui* (27 to 31 by 16 to 18 microns; versus 25 to 29 by 14 to 16 microns in other species). Body size in our specimens ranges from 400 to 740 by 150 to 250 microns which is within the range given by other authors. The bifurcation of the ceca is slightly anterior to the middle of body, the gonotyl slightly behind. Other measurements are too variable to be of any practical value but they fall within the range given by other authors.

Small numbers of *Haplorchis* were obtained from 2 laboratory-bred kittens and 1 laboratory-bred dog fed on cysts dissected from *Gambusia* subsequent to exposure of this fish to selected cercariae from naturally infected *Pirenella conica* taken at El Burg. These specimens closely resemble *H. pumilio* in body shape, short, broad ceca and relatively large eggs but in none of them could the row of spines on the gonotyl be observed. Spines may have been lost in the course of preparation of specimens. We tentatively identify these as *H. pumilio.* They do not have the claw-like hooks characteristic of *H. yokogawai* (see below) which can usually be seen

even when other spines are missing. Two albino rats fed metacercariae from the same lot of infected *Gambusia* produced no adult *Haplorchis*.

A second species of *Haplorchis*, usually accompanied by *H. pumilio*, identified as *H. yokogawai* (Katsuta, 1932) was found in fewer hosts (Table I). This species is slightly larger (700 to 910 microns) and the fore part of the body is broader and flatter than in *H. pumilio*. Mounted specimens rarely come to rest on their sides since the posterior part of body is broader than deep. The ceca which end near the anterior border of the testis in *H. pumilio* reach to or beyond the posterior end of the testis in *H. yokogawai*. The eggs are smaller than those of *H. pumilio* and the gonotyl (Pl. I, Fig. 2, a–c) is characterized by the presence of a pair of clawlike hooks on a rounded terminal lobe that can be projected from the surface of the body. In some specimens there is a row of very fine spines on the margin of this lobe as well as some very fine spines on its surface. The latter, however, are distinguishable only with difficulty. Internally the gonotyl is elongated and ends in a rounded lobe. It is flanked just behind the protrusible claw-bearing lobe by a pair of postero-laterally directed lobes not clearly seen in many specimens. The gonotyl spines, as described above, fit the description given for *H. yokogawai* by Chen (1936) except that the fine surface spines are less conspicuous and cannot be seen in some specimens. They also fit Gohar's (1934b) *H. pumilio* which, as noted above, is undoubtedly *H. yokogawai* since it also has the long ceca and small eggs characteristic of this species. Africa (1938) figured a pair of chitinized structures suggestive of the clawlike hooks of *H. yokogawai* in *H. vanissima* (from the Philippines) but other spines on the gonotyl are quite different. Abdel Azim (1938) reported this species which he erroneously considered a synonym of *H. milvi* Gohar, 1934 (as was suggested by Chen, 1936) in dogs from Cairo, Alexandria, and Upper Egypt. He gave no basis for his identification; consequently we cannot be certain that he was dealing with the species considered herein to be *H. yokogawai*, although this seems likely.

A third species, *Haplorchis taichui*, is easily recognized by 14 to 20 chitinous bars arranged fan-like on the gonotyl. Abdel Azim reported it from dogs in Cairo and Upper Egypt but failed to find it in dogs in Alexandria. Witenberg (1929) found this species not uncommon in cats and dogs, once in a gull (*Larus*) in Palestine and from the experimental feeding of dogs with various fishes including *Gambusia*. We found single specimens of this parasite in a cat from Giza Province, in a cat from the Yemen and in a dog from Cairo.

Phagicola

Two species of *Phagicola* were found in Egyptian cats (Table I). One is *P. ascolonga* (Witenberg, 1929) Price 1932. The other is very similar to *P. longa* (Ransom, 1920) Price 1932 but differs in so many details that we feel it necessary to consider it a distinct and previously undescribed species, for which we propose the name *Phagicola longicollis*. Abdel Azim (1938) reported *P. ascolonga* and *P. italica* as well as *P. longa* from dogs in Cairo and Alexandria. Since the latter was found so commonly by Abdel Azim and since he gave no basis for his identification, it seems possible that he may have been working with our *P. longicollis* rather than with *P. longa*.

P. ascolonga was found by Witenberg (1929) in dogs and cats in Jerusalem, usually in small numbers, and was experimentally obtained in dogs by feeding them

fish, *Tilapia simonis* and *T. galilae*. It was differentiated from *P. italica* only by the longer appendix of the oral sucker which reaches to the pharynx; according to Witenberg, there are no intermediate conditions. In the Egyptian specimens the oral appendage was uniformly long although in a few specimens the posterior part of it was curled forward so that it did not reach the pharynx.

Phagicola longicollis n. sp. was found in cats more frequently than *P. ascolonga* (Table I) and in greater numbers. This species seems to be closely related to *P. longa* (Ransom, 1920), as redescribed by Witenberg (1929), but differs from that species in several details. The body is much more elongate because of the increased length of the slender, neck-like portion anterior to the genital pore. In *P. longicollis* the pharynx is situated a considerably greater distance from the anterior end than in *P. longa* and the ventral sucker instead of being only slightly over half the body length from the anterior end averages 70 per cent. The suckers are smaller; the ventral one is round and measures 30 to 48 microns in diameter, whereas in *P. longa* it is an elongate structure measuring 70 to 75 microns in length and 50 to 55 microns in width. The oral sucker is armed with 14, sometimes 15, spines as compared with 16 in *P. longa*. The ceca are short, ending anterior to the ovary instead of reaching to the level of the testes, and they bifurcate a considerable distance in front of the genital sac. The principal characters which this species has in common with *P. longa* are the structure of the gonotyls, the transverse folds of the uterus and the follicular character of the vitellaria. *P. longicollis* is easily distinguished from *P. ascolonga* by the shape of the body, by the appendage of the oral sucker which does not extend to the pharynx even though it is very long, by the structure of the gonotyl and by the failure of the uterus to extend behind the testis.

Phagicola longicollis n. sp. (Pl. I, Fig. 3)
(all measurements in microns)

Description: Body shaped like elongated sack, sometimes gradually decreasing in width towards anterior end; more often, more or less abruptly narrowed to genital pore. Cuticle spiny, the scale-like spines decreasing in size posteriorly and disappearing at about level of anterior border of testes. Length 610 to 1000; maximum width 120 to 270. Anterior end with well developed dorsal lip. Mouth surrounded by single circle of 14, sometimes 15, spines; spines about 17 to 18 long with oval bases 15 to 16 long and about 10 wide with blunt, curved tips projecting from them. Oral sucker 40 to 50 in diameter with an elongated, slender appendage 125 to 150 long, often curved or bent S-shaped, reaching to 180 to 250 from anterior end and approximately ½ to ¾ distance to pharynx. Pharynx 32 to 53 in diameter, situated 1/3 to ½ length of body from anterior end, average 42 per cent. Esophagus short, often kinked, about 50 to 70 long. Ceca much larger in diameter than esophagus, ending from shortly behind posterior rim of ventral sucker to midway between this and ovary. Ventral sucker round, usually somewhat smaller than pharynx, 30 to 48 in diameter, sometimes showing a knoblike, protrusible, ventrally-projecting process; situated 60 to 75 per cent of length of body from anterior end, averaging 70 per cent. Genital pore a transverse, slit-like opening immediately anterior to ventral sucker. Genital sac 2 to 2½ times diameter of ventral sucker which is contained in it, provided with two lateral, muscular, bulb-like gonotyls; these gonotyls provided with 6 to 8 refractile, gland-like structures, distended and conspicuous in some specimens, not obvious in others. [Similar structures are shown in Witenberg's (1929) figure of *"Parascotyle longa"* and referred to by him as "chitinous bars" and in Travassos' (1929) figure of *"Parascotyle arnaldoi"* as glandular cells].

Testes oval or nearly round, 60 to 80 in greater diameter, situated short distance from posterior end of body, sometimes contiguous but usually separated by stem of Y-shaped excretory bladder. Seminal vesicle large but of variable size, sometimes apparently single, more often in form of 2 transversely oval bodies lying posterior to ventral sucker and separated by transverse loop of uterus. Cloacal duct and course of sperm duct to genital atrium not observed. Ovary somewhat smaller than testes, anterior to right testis on ventral side. Seminal receptacle slightly larger than ovary, at its left side and partially overlapping it dorsally,

round or oval in shape. Vitelline glands form 6 to 8 large lateral follicles around outer boundaries of testes, farther on ventral than on dorsal side. Uterus occupies area from anterior border of testes and vitellaria to just behind ventral sucker, in 3 or 4 transverse loops; terminal portion of uterus directed from dorsal to ventral side just anterior to ventral sucker; genital atrium entered from left side. Eggs 18 to 20 by 10.

Host: Domestic cat
Location: Intestine
Locality: Egypt
Type specimen: U. S. National Museum Helminthological No. 38169

Stellantchasmus falcatus Onji and Nishio, 1916

This fluke was common. In every host except the shrews it was accompanied by *Haplorchis pumilio,* in all but 1 host by *Heterophyes heterophyes,* and in all but 2 by 1 or both species of *Phagicola.* Witenberg (1929) described this parasite under the name *Diorchitrema pseudocirrata.* He reported it as rare in dogs and cats in Palestine and Abdel Azim (1938) found it in dogs of Cairo and Alexandria. Two other species of *Stellantchasmus, S. amplicaecalis* and *S. formosanus,* described by Katsuta in 1932, are regarded by Chen (1951) as synonyms of *S. falcatus* which he considers the only valid species of the genus.

Stictodora sawakinensis Looss, 1899

One specimen of this parasite was removed from a cat in Cairo. This species was first described from a gull (*Larus*) on the Egyptian coast. Witenberg (1929) gave an emended description and reported the fluke as occurring rarely in dogs and cats in Palestine, always in small numbers. He found it once in a puffin (*Puffinis kuhli*) taken at Suez, and Balozet and Callot (1938) reported it from a duck (*Anas platyrhynchus*) in Tunis.

Martin and Kuntz (1955) obtained a fluke which they described as *Stictodora tridactyla,* from chicks experimentally fed on fish, *Aphanius fasciatus* and *Gambusia* sp. The *A. fasciatus* had been found naturally infected on the Mediterranean coast of Egypt; the *Gambusia* had been experimentally infected with cercariae from *Pirenella conica* collected at the same locality.

Pygidiopsis genata Looss, 1907

Looss described this parasite from a pelican in Egypt. Subsequently it was reported from kites and pelicans by Gohar (1934a, 1935). Abdel Azim (1938) found it abundant in Egyptian dogs. It has a wide range of hosts and an extensive geographic distribution (Ciurea, 1924; Linton, 1928; Faust and Nishigori, 1926; Africa and Garcia, 1935; Witenberg, 1929; Balozet and Callot, 1928; Abdel Azim, 1938; Onji and Nishio, 1924; Yosikawa, Miyata and Uesugi, 1940; Travassos, 1929; Yamaguti, 1939 and Refuerzo and Garcia, 1937). *Pygidiopsis* was taken from many cats and a number of other hosts (Table I). In the majority of cats and dogs it was accompanied by *Heterophyes heterophyes, Haplorchis pumilio* and one or both species of *Phagicola.*

Echinochasmus liliputanus Looss, 1896

This small echinostome was described by Looss (1896) from a hawk (*Pernis apivorus*) and two kites (*Milvus migrans*) in Egypt, in small numbers. It was later redescribed by Witenberg (1932) who found it in cats as well as in a heron (*Ardea cincerea*) in Palestine. We found it in cats from scattered localities.

Stephanoprora denticulatoides Isaïchikoff, 1925

A parasite tentatively identified as *S. denticulatoides* was found in 2 of 16 cats from the Mediterranean coast. It is the only *Stephanoprora* reported from mammals. Isaïchikoff (1925) found this species common in dogs in the city of Kerch, Crimea. However, the specimens from the Egyptian cats differ consistently from Isaïchikoff's description of *denticulatoides* in several respects and no *Stephanoprora* has thus far been reported from dogs in Egypt, in spite of the examination of 100 dogs in Alexandria by Abdel Azim. Because of these facts and present uncertainty concerning speciation in this genus, the following description of specimens seems desirable:

Description: (All measurements in microns) Body length 3300 to 4200; maximum width at level of ventral sucker, 320 to 410. Other widths: cephalic collar 250 to 350; neck immediately behind collar, 188 to 245; at level of posterior testes, 210 to 320; of posterior fourth of body, 168 to 235. Other measurements: transverse diameter of oral sucker, 92 to 112; prepharynx, 66 to 118; pharynx usually longer than wide, 104 to 116 long by 100 to 122 wide; ventral sucker 910 to 1230 from anterior end (27 to 29 per cent of body length), usually slightly longer than wide, 200 to 240 long by 203 to 230 wide. Collar spines 22 in number, dorsal and lateral spines 58 to 67 long and 18 to 22 broad at base; 2 ventral "corner" spines set back a little, smaller, last one 39 to 46 long, the second one 43 to 56. Esophagus long, bifurcation of ceca 700 to 945 from anterior end. Genital pore just behind bifurcation. Testes tandem, not quite touching; middle of anterior testis at or near equator of body, slightly more anterior in smaller worms and slightly more posterior in larger ones. Anterior testis slightly elongated, 270 to 330 long and 155 to 190 broad. Total testicular region 560 to 630, 1/6 or less body length. Cirrus pouch 200 to 222 long and 75 to 118 wide, reaching to middle of ventral sucker or slightly beyond. Ovary small, round, 85 to 92 in diameter, separated from anterior testis by 132 to 148, this space occupied by proximal part of uterus, vitelline reservoir and oötype surrounded by large Mehlis gland. Vitellaria extend from 50 to 80 from posterior end of body to about middle of anterior testis, although in one specimen they reach barely beyond the posterior border of this testis. There are only a few follicles lateral to anterior testis. Uterus undulant rather than in distinct loops, containing from 5 to 17 eggs except in the smallest specimen in which the uterus is empty. Eggs in uterus of mounted specimens 76 to 52.

Isaïchikoff (1925) considered *S. denticulatoides* very close to *S. denticulata* of European birds, from which he differentiated it on the basis of large collar spines; larger body, suckers, collar, ovary and testes; and more anterior extent of the vitellaria than is usual in *denticulata*. From one or another of the other species he differentiated *denticulatoides* on the basis of anterior extent of the vitellaria to the middle of anterior testis, position of anterior testis at equator of body; long prepharynx and consequently more posterior position of the pharynx; number and arrangement of collar spines; and short distance between ovary and anterior testis. Beaver (1937b) stated that *denticulatoides* was apparently identical with *S. polycestus* (sic), though somewhat smaller. In disagreement with Odhner (1910), he differentiated *S. polycesta* from *denticulata* on the basis of extent of the vitellaria to middle of the anterior testis (although Dietz, 1910, stated that in one specimen of *denticulata* the vitellaria reached to this level); more extensive uterus; and relatively smaller suckers and head crown. *S. reynoldi* Bhalarao, 1926 and *S. merulae* Yamaguti, 1933, each described from a single specimen and both synonymized with *polycesta* by Beaver, agree with *polycesta* in respect to vitellaria and uterus, and this is also true of *S. magniovata* Yamaguti, 1939. On the other hand, *S. denticulatoides,* although agreeing with *polycesta* in the anterior extent of vitellaria, agrees with *denticulata* in its short uterus and few eggs, and also in its rather large suckers and collar spines.

Specimens from Egyptian cats agree with *denticulatoides* in all characters by which Isaïchikoff distinguished this species from species other than *denticulata*, except for a considerably greater distance between the ovary and anterior testis. With respect to the characters used to distinguish *S. denticulatoides* from *S. denticulata*, our specimens agree with *denticulata* in small size of ovary and they are intermediate in size of suckers (absolute and relative) and of the testes. They differ from *polycesta* mainly in the smaller extent of the uterus, with few eggs; the smaller ovary and testes; more posterior position of the testes; and the much more slender post-testicular region of the body. They differ from *denticulatoides* also in this last respect and also in the smaller maximum diameter (at the level of the ventral sucker), greater distance of the genital pore from the anterior end, considerably smaller suckers and pharynx, broader collar spines and greater distance between ovary and anterior testis.

In view of the very great variations which Beaver (1937a) observed in his careful study of *Echinostoma revolutum*, a member of the same family, depending on age, method of fixation, and host, and the similar variations that we and others have observed in *Heterophyes heterophyes*, we are doubtful of the validity of many of the described species of *Stephanoprora*. In comparing our specimens from cats with Isaïchikoff's from dogs it is interesting to note that there are the same striking differences in slenderness of body and size of suckers that exist between *H. heterophyes* from cats as compared with those from dogs. Since *Stephanoprora* is parasitic in birds of wide range and migratory habits, it would be expected that the species in them would be widely distributed geographically. Possibly some of the differences observed in specimens from different localities and hosts may be dependent not only on such factors as age, fixation, definitive host, acquired resistance, and crowding, but also on development in different first and/or second intermediate hosts. There seems little hope of settling the matter of speciation in this genus until the life cycle has been worked out and experimental infections made as was done by Beaver for *Echinostoma revolutum* and by Witenberg for *Heterophyes heterophyes*. Meanwhile it seems best tentatively to retain such forms as *polycesta*, *denticulata* and *denticulatoides* as separate species to avoid further confusion, although it will still be desirable to give descriptions of forms from new hosts and localities, such as the one described here, whenever these appear to have consistent differences.

Mesostephanus appendiculatus

Although this fluke has not been previously reported from Egypt, we found it frequently in both cats and dogs and also in one fox (Table I). Its geographic distribution suggests possible limitation to fresh water intermediate hosts. Since Abdel Azim (1938) reported *Prohemistomum vivax* commonly present in dogs in Cairo and Upper Egypt but failed to report *Mesostephanus appendiculatus*, it seems possible that there may have been a misidentification.

Cynodiplostomum namrui n. sp. (Pl. II, Figs. 1–3)

In 7 of 48 cats and in 2 of 3 dogs (Table I) we found a species of *Cynodiplostomum* which is unlike *C. azimi* Gohar, 1933, the only member of the genus hitherto known. We, therefore, regard this a new species for which we propose the name *C. namrui*, after the U. S. Naval Medical Research Unit No. 3 in Cairo.

The specimens in the dogs did not differ in size or anatomy from those in cats. It is possible that the *"Diplostomum tergenna"* (= *D. tregenna*) reported by Abdel Azim (1938) in 5 per cent of Cairo dogs may have been this species, although in size *D. tregenna* more closely resembles *C. azimi*.

The genus *Cynodiplostomum* was erected by Dubois (1936) for a trematode taken from an Egyptian dog and described by Gohar (1933) as *Diplostomum azimi*. Because of the limitation of the vitellaria to the forebody Dubois placed it in the subfamily Alariinae. Dubois later (1938) differentiated it from *Alaria* on the basis of the small, circular holdfast organ situated behind the middle of the forebody, and the lateral position of the ovary, situated opposite the assymetrically developed anterior testis. Our species agrees with these characteristics except that the holdfast organ is broadly oval instead of round and not particularly small. The structure of terminal portions of the reproductive canals and the bursa copulatrix were not described for *C. azimi* but Gohar's figure suggests that the structure may be similar to that described for *C. namrui* below.

Description: (All measurements in microns) Body 750 to 1200 long with maximum width of 250 to 450. No clear demarcation between fore- and hindbody in most specimens. Forebody, measured to ventral union of lateral flanges, 680 to 860 long, over twice and usually 3 times length of hindbody (250 to 390) as measured from this point. Body bent dorsally at junction of fore- and hindbody. Oral sucker broader than long, 84 to 107 by 67 to 83; pharynx longer than broad, 66 to 77 by 60 to 68; no prepharynx or esophagus. Lateral suckers well developed. Ventral sucker broader than long, 68 to 75 by 72 to 97, slightly smaller than oral sucker, situated approximately middle of length of forebody, 310 to 450 from anterior end. Holdfast organ oval, 175 to 200 broad and 210 to 230 long, not clearly demarcated posteriorly and situated near posterior end of forebody and separated from ventral sucker by 80 to 140. Ceca long, ending near or just behind end of posterior testis.

Posterior testis situated toward anterior portion of hindbody, consisting of 2 elongated lateral lobes occupying most of thickness of body dorsoventrally and connected by a rather narrow bridge posterior and dorsal in position; lateral lobes about 165 to 210 in length and 100 to 150 in width. Anterior testis of variable shape and size, consisting of single, laterally placed lobe usually somewhat larger than one of lateral lobes of posterior testis; situated on left in most specimens but on right in others. Seminal vesicle situated between lobes of posterior testis, dorsal to vagina, variable in size. Sperm duct opens into ejaculatory duct from dorsal side. Ovary approximately round, 50 to 70 in diameter, situated near middle of body width or on side opposite anterior testis, overlapping this ventrally. Mehlis' gland on same side as ovary, posterior to it and more dorsally situated. Laurer's canal a narrow duct opening at level of seminal vesicle. No seminal receptacle observed. Uterus a broad, thin-walled tube passing ventrad between ovary and vitelline reservoir, then turning posteriad and entering upper end of ejaculatory duct. Vitellaria from level of ventral sucker to posterior end of forebody on dorsal side, especially dense dorsal to holdfast organ. Vitelline reservoir 3-pronged, 2 prongs directed laterally and forward as the vitelline ducts, the third prong directed dorsad and somewhat posteriad towards Mehlis' gland. Ejaculatory duct large and very heavy-walled, receiving uterus anteriorly, sperm duct on its mid-dorsal side and opening into a shallow disc-shaped ejaculatory pouch. Immediately behind this, at upper end of large, thick walled copulatory bursa, is a well developed sphincter. Excretory pore on ventral side about 70 from posterior end of body. Eggs large, 1 to 4 in number, measuring 100 to 110 by 60 to 67.

Hosts: Cat and dog
Location: Small intestine
Locality: Egypt
Type specimen: U. S. National Museum Helminthological No. 38168

C. namrui is only 1/2 to 1/3 the length of *C. azimi* and the width 1/3 to 1/5, a character that is constant in dozens of specimens from both cats and dogs. The more elongate shape of the parasite is due to the much greater length of the forebody relative to the hindbody. Relative size of the suckers is approximately the same as in *C. azimi* but only half as large. The holdfast organ is distinctly longer

than broad and is more posteriorly situated. The vitellaria are less dense and consist of larger follicles. Unless Gohar's specimen, experimentally obtained, was aberrant and incorrectly figured, it does not seem reasonable to consider these two co-specific, even in view of the great variations that occur in these flukes.

SUMMARY

Ten heterophyids were identified in a collection of flukes obtained from 48 cats, 3 dogs, a fox (*Vulpes vulpes*), 2 shrews (*Crocidura olivieri*), a rat (*Rattus rattus*), and several birds (5 kites, *Milvus migrans;* an osprey; and a domestic duck) in Lower Egypt. *Heterophyes heterophyes* occurred in the majority of cats and dogs, in the fox and in man. *Heterophyes aequalis,* believed to be synonymous with *H. dispar,* was common in the majority of mammals and was obtained experimentally in cats and dogs. Three species of *Haplorchis, H. pumilio, H. yokogawai* and *H. taichui,* were encountered. *H. pumilio* was found in the majority of cats and dogs, in the fox, in both shrews and in 1 kite, and experimentally in cats and dogs. *H. yokogawai* was found in approximately 1/3 of dogs and cats and in 1 kite, whereas only a single *H. taichui* occurred in each of 2 cats. *Stellantchasmus falcatus* was found in the majority of cats, 1 dog and 1 shrew. Two species of *Phagicola* were found, *P. ascolonga* and *P. longicollis* n. sp., both in approximately 1/3 of cats but not in other hosts. *Pygidiopsis genata* was obtained from about 1/2 of the cats and dogs, in the fox and from 1 shrew, in the rat and in 3 kites. One *Stictodora sawakinensis* was removed from a cat.

In addition to the heterophyids, *Echinochasmus liliputanus* was recovered from approximately 1/4 of cats, 2 dogs and the fox. A species of *Stephanoprora* agreeing with *S. denticulatoides* in most respects was present in 2 cats. *Mesostephanus appendiculatus* was present in more than 1/3 of the cats and dogs and in the fox. *Cynodiplostomum namrui* n. sp., was obtained from 17 of 48 cats and 2 dogs. The relationships of different species in the genera *Heterophyes, Haplorchis* and *Stephanoprora* are discussed in some detail.

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EXPLANATION OF FIGURES

PLATE I

FIG. 1. Gonotyl of *Haplorchis pumilio* projecting from side of body, in lateral view. Three eggs also shown. Note incomplete crown of odd-shaped hooks or spines, and clusters of very small spines, actually at openings of male and female ducts.

PLATE I

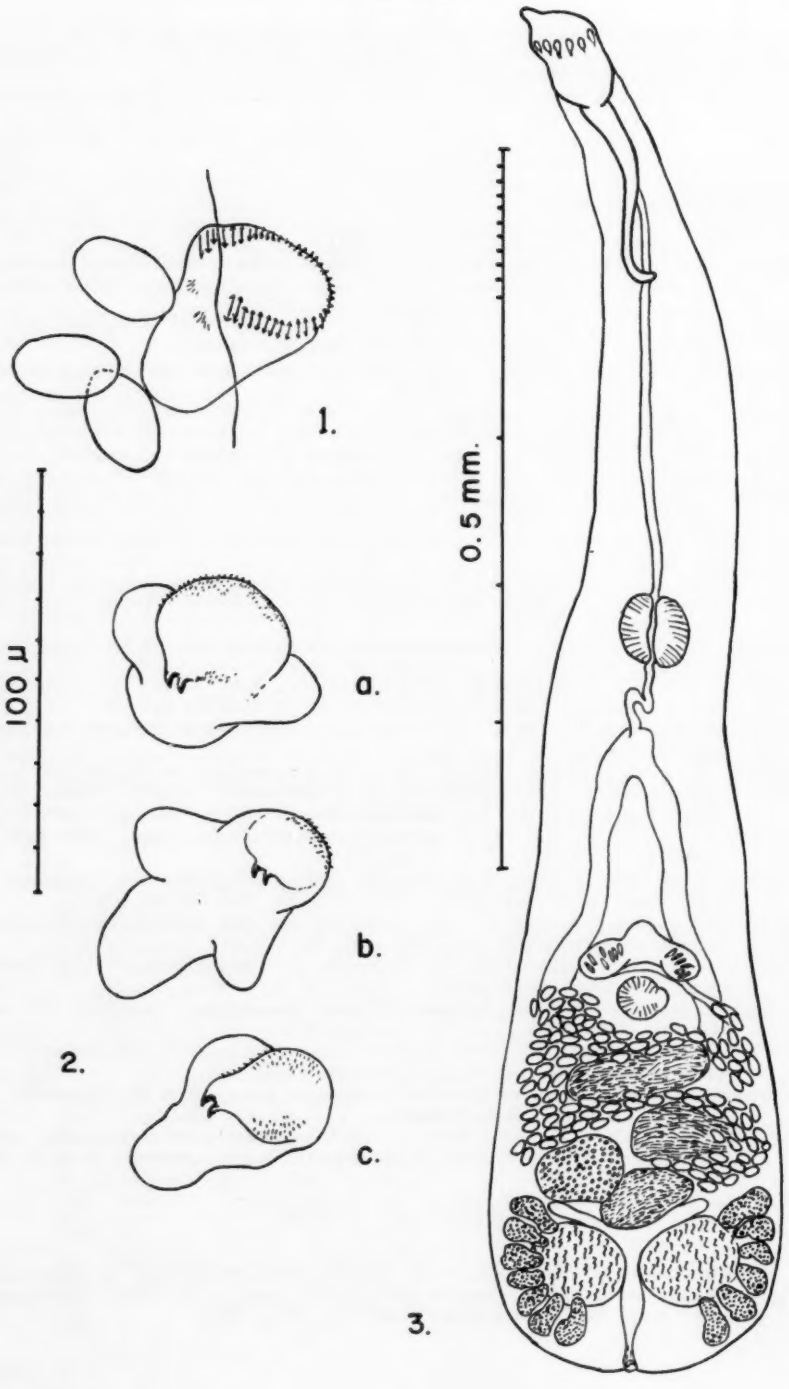
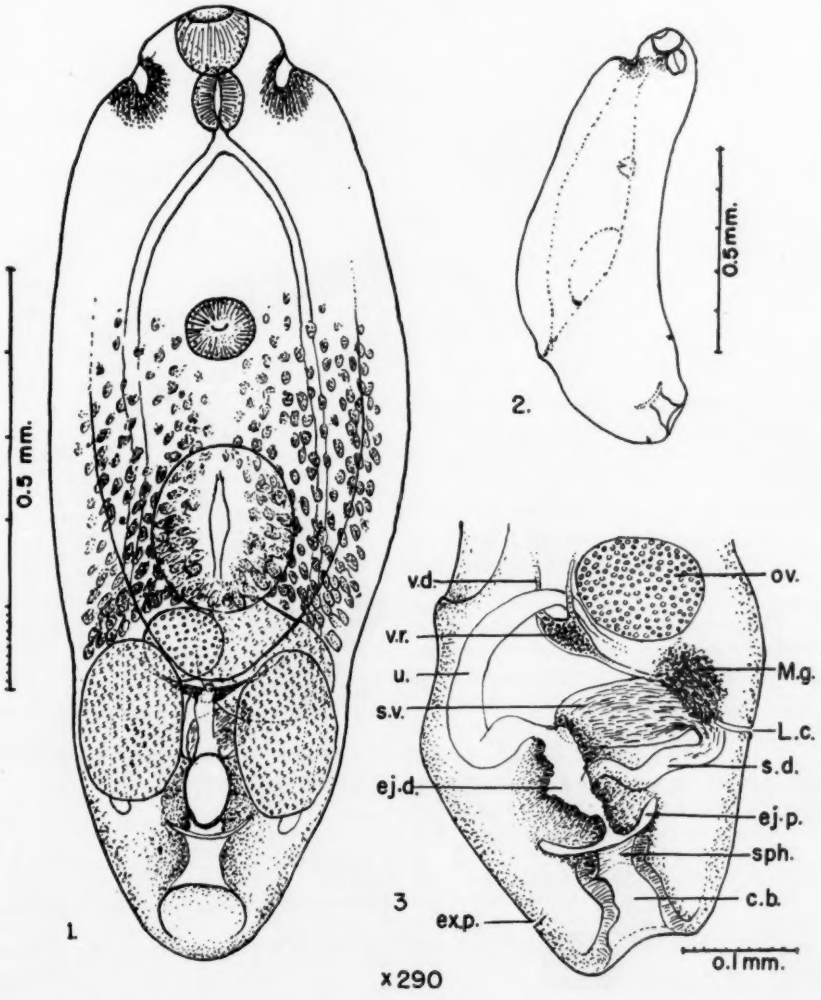


FIG. 2. Three views of gonotyl of *Haplorchis yokogawai* as seen from ventral side. Only the lobe bearing the pair of clawlike hooks projects from the surface.
FIG. 3. Adult, *Phagicola longicollis,* n. sp., ventral view.

PLATE II

FIG. 1. Adult, *Cynodiplostomum namrui,* n. sp., ventral view.
FIG. 2. Same, outline of body in lateral view.
FIG. 3. Same, hindbody.
Abbreviations: *c.b.,* copulatory bursa; *ej.d.,* ejaculatory duct; *ej.p.,* ejaculatory pouch; *ex.p.,* excretory pore; *L.c.,* Laurer's canal; *M.g.,* Mehlis' gland; *ov.,* ovary; *s.d.,* sperm duct; *sph.,* sphincter; *s.v.,* seminal vesicle; *u.,* uterus; *v.d.,* vitelline duct; *v.r.,* vitelline reservoir.

PLATE II



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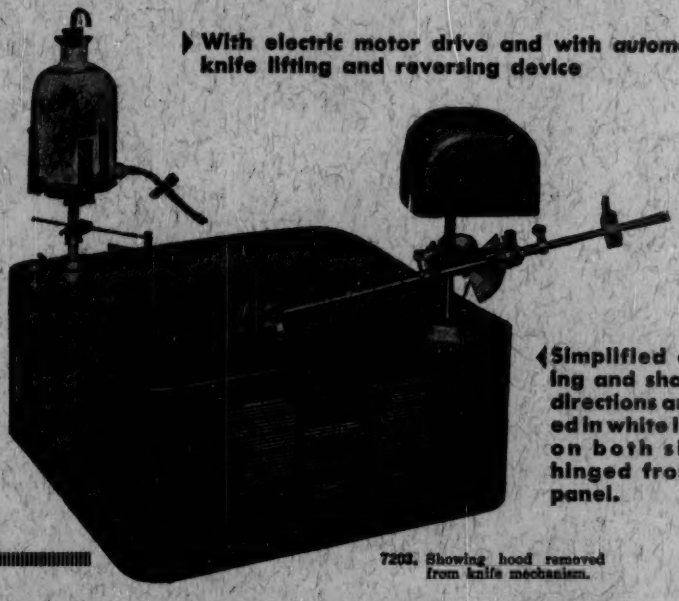

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